Universidade do Minho
Escola de Engenharia

Ana Luísa Dominguez de Matos

Fructooligosaccharides production - Ingredients for functional food

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UMinho | 2013

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Tese de Doutoramento
Doutoramento em Engenharia Química e Biológica

Trabalho efetuado sob a orientação do
Professor Doutor José António Couto Teixeira
e do
Professor Doutor Nelson Manuel Viana da Silva Lima

Fevereiro 2013

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Título tese: Fructooligosaccharides production – Ingredients for functional food

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Ano de conclusão: 2013

Doutoramento em Engenharia Química e Biológica

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Universidade do Minho, 7 de Fevereiro de 2013

AGRADECIMENTOS

Ao finalizar esta tese, gostaria de agradecer a todos aqueles que, de uma forma ou de outra, me acompanharam a apoiaram ao longo destes últimos anos. Muitas vezes caímos no erro de não mostrar aos que nos rodeiam a importância que estes têm na nossa vida, por isso, achei que não devia perder a oportunidade de deixar o meu agradecimento a todos aqueles que tiveram e têm papéis preponderantes na minha vida.

Gostaria de agradecer aos meus orientadores científicos Professor José Teixeira e Professor Nelson Lima por todo o apoio concedido ao longo destes anos. Indiscutivelmente as suas qualidades científicas, disponibilidade e estímulo foram fundamentais para a realização deste trabalho. É ainda devido um agradecimento adicional ao Professor José Teixeira, na qualidade de director do Centro de Engenharia Biológica, por me ter acolhido como investigadora neste centro.

Gostaria de agradecer a toda a equipa BIOLIFE que em tanto contribuiu para o bom termo deste trabalho. À Lígia Rodrigues por todo o apoio, ensinamentos, amizade e disponibilidade que, sem sombra de dúvida, foram indispensáveis na realização desta tese. À Isabel Rocha e ao Duarte Torres também agradeço por todo o apoio e amizade demonstrados e à Clarisse Nobre pela ajuda prestada em algumas tarefas experimentais.

Ao António Peres agradeço todo apoio prestado no desenho experimental e no tratamento estatístico dos resultados.

À Isabel Santos por toda a disponibilidade e apoio sempre prestados na Micoteca.

Ao Telmo Adrego agradeço o apoio prestado na análise económica do processo industrial.

A todos os meus colegas e amigos do Laboratório de Fermentações e do CEB. Foram muitos os amigos com quem tive o privilégio de viver agradáveis momentos e a todos vocês agradeço a amizade sempre demonstrada.

A todos quantos trabalham no DEB agradeço a amizade. Um reconhecimento especial à Madalena Vieira pelo seu valioso apoio técnico no HPLC, ao Sr. Santos pela sua disponibilidade, apoio e boa disposição e ao Paulino Amorim pela amizade sempre demonstrada.

À Olívia Pereira queria deixar um agradecimento especial por ter feito nascer em mim o “bichinho” da investigação e acima de tudo por toda a amizade e apoio sempre demonstrados.

À Dra. Isabel Oliveira, mais do que minha médica, uma amiga sempre disponível. Obrigada pelos merecidos “puxões de orelha”, por me relembrar de vez em quando quais devem ser as minhas prioridades e por todas as palavras de amizade e incentivo. Obrigada por tudo...

A todos os meus queridos amigos os meus sentidos agradecimentos. À Filipa pela longa e incondicional amizade, apoio e por estar sempre lá nos momentos bons e “menos bons”... Uma verdadeira “mana do coração”. Ao Rui (mesmo estando do outro lado do mundo) obrigada por toda a amizade, apoio e eterna boa disposição. À Gi e à Bina deixo um agradecimento por toda a amizade e apoio e por todos os nossos momentos diários de boa disposição. Ao Daniel e à Denise expresso os meus sinceros agradecimentos pela sua incondicional amizade e apoio.

À Fundação para a Ciência e a Tecnologia pelo apoio concedido para a realização deste trabalho através da bolsa SFRH/BD/23083/2005.

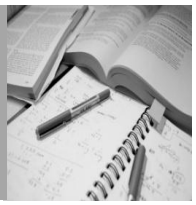
Para a minha família deixo os mais sentidos agradecimentos e reconhecimento. Sem vocês não seria quem sou. Obrigado por simplesmente estarem aí.... Aos meus Pais, Glória e Jorge, pelas oportunidades que sempre me proporcionaram, carinho e apoio. À minha tia, Luísa, por toda a dedicação e carinho. Aos meus manos, André e Paulo, pelo eterno carinho e apoio... realmente não consigo imaginar a minha vida sem vocês. Às minhas cunhadas, Carla e Nassima, pelo carinho e amizade. Aos meus sobrinhos, Francisco, Tiago, Guilherme, Sofia e Maria por todos os momentos de alegria. À D. Margarida e à Maria João pelo carinho com que me “acolheram” na família. À minha Mãe devo ainda um reconhecimento acrescido pela infindável paciência, carinho, estímulo e as minhas desculpas por todas as preocupações... Devo-lhe grande parte do que sou por isso este trabalho também lhe pertence.

Ao Paulo, por todo o amor, amizade, apoio e uma grande dose de paciência. Obrigada por estares a meu lado e por fazeres tudo parecer mais simples... “you’re my destiny”.

Por fim gostaria de dedicar esta tese à memória dos meus avós Martinho e Maria, por tudo que sempre significaram e significam na minha vida...

Ana

ABSTRACT



FRUCTOOLIGOSACCHARIDES PRODUCTION – INGREDIENTS FOR FUNCTIONAL FOOD

This thesis addresses the development and optimization of a fermentation process for the production of fructooligosaccharides (FOS) by fungi. FOS are prebiotics with numerous health benefits within which the improvement of gut microbiota balance can be highlighted, playing a key role in individual health. Conventionally, FOS production is a two-stage process that requires an enzyme production/purification step in order to proceed with the chemical reaction itself. FOS production by fungi in bioreactors in a one-stage process is an emergent alternative, since the use of whole cell as the biocatalyst avoids the need for FOS-producing enzyme purification from the cell extract, thus enabling a more competitive less expensive process. Therefore, the main purpose of this thesis was to develop and optimize a one-stage fermentation process for a more competitive FOS production towards its industrial implementation. Optimization of fermentation conditions to maximize FOS production via sucrose fermentation by *Aureobasidium pullulans* through response surface methodology, using a compilation of mathematical and statistical techniques, was performed and a significant improvement of the total FOS production yield by using a one-stage process was obtained.

Although several microorganisms have been reported to have transfructosylation activity due to fructosyltransferase (FTase) and/or fructofuranosidase (FFase) activities, the search for other fungi with higher transfructosylation activity remains a challenge. By means of a presumptive and indirect colorimetric plate assay developed for the evaluation of transfructosylation activity in fungi, several fungal strains belonging to the *Aspergillus* and *Penicillium* species were identified, showing potential as FOS-production microorganisms.

An enzymatic extraction procedure was established to determine the transfructosylation and hydrolyzing activities of the enzymes in the extracellular, intracellular and periplasmic spaces of *A. pullulans*. Extracellular enzymes were

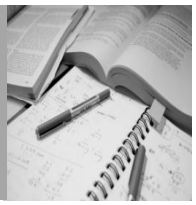
found to have the higher transfructosylation activity, supporting the idea of developing a one-stage FOS production process.

Fed-batch sucrose fermentations by *A. pullulans* with two sucrose feeding profiles (constant feeding and linear feeding) were conducted in order to increase FOS production. A slight increase in FOS production yield was achieved with sucrose linear feeding profile, compared to the batch production process, however FOS productivity decreased significantly. The main advantage to operate in fed-batch mode with a linear feeding profile was shown to be the production of fructofuranosyl nystose (GF4), that can add commercial value to the FOS mixture obtained, since GF4 may exert a prebiotic effect in more distal colonic regions compared with the lower-molecular-weight FOS.

The scale-up of the one-stage batch FOS production process from sucrose by *A. pullulans*, to a semi-pilot scale, was performed. Similar FOS production yield, productivity, composition and kinetics were obtained by a 25 times volume increase scaling up procedure, suggesting that the one-stage batch process has potential to be used at an industrial level. Moreover, based on the process design and economics of an industrial unit built to produce 10.000 tons/year of FOS using the here developed production process, it was demonstrated that lower operational costs can be achieved, when compared to the current two-stage FOS production process. An estimated investment of 20 M€ in a FOS industrial unit can achieve a NPV of 71,5 M€, with a IRR of 45,18 % and ROI of 5 years, presenting itself as a very attractive business opportunity.

Keywords: fructooligosaccharides, *Aureobasidium pullulans*, optimization of the fermentation conditions, production yield, productivity, fructosyltransferase, fructofuranosidase, transfructosylation, one-stage fermentation process.

SUMÁRIO



PRODUÇÃO DE FRUTO-OLIGOSSACÁRIDOS – INGREDIENTES PARA ALIMENTOS FUNCIONAIS

Esta tese aborda o desenvolvimento e otimização de um processo de fermentação para a produção de fruto-oligossacáridos (FOS) por fungos. Os FOS são pré-bióticos com inúmeros benefícios para saúde, dos quais se pode destacar o equilíbrio da flora intestinal, desempenhando um papel fundamental na saúde do indivíduo. Convencionalmente, a produção de FOS é um processo em duas etapas, que requer uma etapa de produção/purificação enzimática para que a reação química ocorra. A produção de FOS por fungos em biorreatores, tendo por base um processo de produção por uma só etapa, é uma alternativa emergente, dado que a utilização das células inteiras como biocatalisador evita a purificação das enzimas produtoras de FOS do extrato celular, originando um processo mais competitivo e menos dispendioso. Por esse motivo, o principal objetivo desta tese foi o desenvolvimento e otimização de um processo de fermentação para produção de FOS mais competitivo, numa só etapa, com vista à sua implementação industrial. A otimização das condições de fermentação para maximização da produção de FOS por fermentação de sacarose por *Aureobasidium pullulans* foi efetuada usando a metodologia de otimização fatorial, com recurso a uma série de ferramentas matemáticas e estatísticas, tendo-se alcançado uma melhoria significativa do rendimento global de produção de FOS por *A. pullulans* com o processo de produção em uma só etapa.

Vários microrganismos são reportados como tendo atividade de transfrutosilação por intermédio das enzimas frutosiltransferase (FTase) e / ou frutofuranosidase (FFase). No entanto, a procura de outros fungos com atividade de transfrutosilação mais elevada permanece um desafio. Através de um ensaio colorimétrico em placa, presuntivo e indireto, desenvolvido para a avaliação da atividade de transfrutosilação em fungos, várias estirpes de fungos dos géneros *Aspergillus* e *Penicillium* spp foram identificados como potenciais microrganismos produtores de FOS.

Um procedimento de extração enzimática foi estabelecido para determinar as atividades de transfrutosilação e hidrólise das enzimas nos espaços extracelulares, intracelulares e periplasmático de *A. pullulans*. As enzimas extracelulares demonstraram ter a atividade de transfrutosilação mais elevada, reforçando a ideia do desenvolvimento de um processo de produção numa só etapa.

Processos de fermentação semi-contínua de sacarose por *A. pullulans,* com dois perfis de alimentação (alimentação constante e alimentação linear) foram realizados com a finalidade de aumentar a produção de FOS. Um ligeiro aumento no rendimento de produção de FOS foi conseguido com o perfil de alimentação linear de sacarose, em comparação com o processo de produção descontínuo. No entanto, a produtividade de FOS diminuiu significativamente. A principal vantagem de operar em modo semi-contínuo com um perfil de alimentação linear é produzir frutofuranosil nistose (GF4), que pode incrementar o interesse comercial da mistura de FOS obtida, uma vez que o GF4 pode exercer um efeito pré-biótico em regiões mais distais do cólon, em comparação com os FOS de menor peso molecular.

Um aumento de escala no processo de produção de FOS numa só etapa, por fermentação de sacarose por *A. pullulans*, a uma escala semi-piloto, foi realizado e com um aumento de escala de 25 vezes, foram obtidos semelhantes rendimentos de produção de FOS, composição, produtividade e cinéticas de fermentação, sugerindo que o processo numa só etapa tem potencial para ser usado a um nível industrial. Além disso, com base no processo de conceção e economia de uma unidade industrial para a produção de 10.000 ton/ano de FOS, tendo por base o processo de produção desenvolvido nesta tese, menores custos de operação podem ser obtidos, quando comparados com o atual processo de produção de FOS em duas etapas. Um investimento estimado de 20 M€ numa unidade industrial de FOS pode promover um VAL de 71,5 M €, com uma TIR de 45,18% e um tempo de retorno do investimento de 5 anos, apresentando-se como uma oportunidade de negócio muito atrativa.

Palavras-chave: fruto-oligossacáridos, *Aureobasidium pullulans*, otimização das condições de fermentação, rendimento de produção, produtividade, frutosiltransferase, frutofuranosidase, transfrutosilação, processo de fermentação numa só etapa.

SCIENTIFIC OUTPUT



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TABLE OF CONTENTS, LIST OF FIGURES, LIS OF TABLES AND LIST OF SYMBOLS



TABLE OF CONTENTS

GENERAL INTRODUCTION 1

CONTEXT AND MOTIVATION 2

RESEARCH AIMS 3

OUTLINE OF THE THESIS 4

REFERENCES 5

CHAPTER 1: FOS - INGREDIENTS FOR FUNCTIONAL FOOD 7

1.1. INTRODUCTION 8

1.2. FUNCTIONAL FOODS 9

1.3. PROBIOTICS 11

1.4. PREBIOTICS 12

1.5. SYNBIOTICS 14

1.6. FRUCTOOLIGOSACCHARIDES AS PREBIOTICS 15

1.6.1. FOS occurrence 16

1.6.2. FOS production 17

1.6.2.1. FOS microbial production – transfructosylation enzymes 17

1.6.2.2. FOS production by submerged fermentation (SmF) 19

1.6.2.3. FOS production by solid state fermentation (SSF) 21

1.6.2.4. Production of high-content FOS 22

1.6.3. FOS functional properties 23

1.6.4. FOS market data 28

1.7. CONCLUSIONS 30

1.8. REFERENCES 30

CHAPTER 2: SCREENING OF FUNGI WITH TRANSFRUCTOSYLATION ACTIVITY 41

2.1. INTRODUCTION 42

2.2. MATERIALS AND METHODS 44

2.2.1. Reagents and enzymes 44

2.2.2. Microorganism and culture conditions 44

2.2.3. Screening procedure 44

2.3. RESULTS 45

2.4. DISCUSSION 48

2.5. CONCLUSION 49

2.6. REFERENCES 50

CHAPTER 3: OPTIMIZATION OF FOS PRODUCTION BY AUREOBASIDIUM PULLULANS USING RESPONSE SURFACE METHODOLOGY 53

3.1. INTRODUCTION 54

3.2. MATERIALS AND METHODS 55

3.2.1. Microorganism and culture conditions 55

3.2.2. Experimental design and data analysis 55

3.2.3. Sugar analysis 58

3.2.4. Cell growth 58

3.3. RESULTS 59

3.3.1. Effects of the fermentation conditions on FOS production 59

3.4. DISCUSSION 64

3.5. CONCLUSION 66

3.6. REFERENCES 67

CHAPTER 4: TRANSFRUCTOSYLATION ACTIVITY OF THE ENZYMES PRODUCED BY *AUREOBASIDIUM PULLULANS* 71

4.1. INTRODUCTION 72

4.2. MATERIALS AND METHODS 73

4.2.1. Microorganism and culture conditions 73

4.2.2. Enzyme extraction procedure 73
4.2.3. Bright field microscopy 76
4.2.4. Enzymatic activity 76
4.2.5. Total protein determination – Microplate procedure 76
4.3. RESULTS 77
4.4. DISCUSSION 78
4.5. CONCLUSION 79
4.6. REFERENCES 80

CHAPTER 5: FOS PRODUCTION BY FED-BATCH FERMENTATION OF SUCROSE BY *AUREOBASIDIUM PULLULANS* 83
5.1. INTRODUCTION 84
5.2. MATERIALS AND METHODS 86
5.2.1. Microorganism and culture conditions 86
5.2.2. Fermentation Operation 86
5.2.3. Sugar analysis 87
5.2.4. Cell growth 87
5.2.5. Enzymatic activity 87
5.2.6. FOS production yield and productivity determination 88
5.3. RESULTS 88
5.3.1. “Fed-Batch 1” – Constant feeding profile 88
5.3.2. “Fed-Batch 2” – Linear feeding profile 90
5.4. DISCUSSION 92
5.5. CONCLUSION 94
5.6. REFERENCES 94

CHAPTER 6: SCALE-UP OF A ONE-STAGE FOS PRODUCTION PROCESS 97
6.1. INTRODUCTION 98
6.2. MATERIALS AND METHODS 99
6.2.1. Microorganism and culture conditions 99

6.2.2. Fermentation Operation.....100
6.2.3. Sugar analysis.....100
6.2.4. Cell growth.....101
6.2.5. Process design and economics.....101
6.3. RESULTS.....101
6.3.1. FOS production on a semi-pilot scale.....101
6.3.2. FOS industrial production design and economics.....103
6.4. DISCUSSION.....106
6.5. CONCLUSION.....107
6.6. REFERENCES.....107

CHAPTER 7: GENERAL CONCLUSIONS AND FUTURE WORK.....109
7.1. CONCLUSIONS.....110
7.2. RECOMMENDATIONS FOR FUTURE WORK.....111

LIST OF FIGURES

CHAPTER 1

Figure 1.1. Structures of 1-kestose (GF2) (left), nystose (GF3) (center), and fructofuranosyl nystose (GF4) (right). 16

Figure 1.2. Schematic representation of the two stage FOS production process by SmF (From Sangeetha *et al*. 2005b). 19

Figure 1.3. Interactions of prebiotics, probiotics and immune system in the intestinal mucosa. Prebiotics promote the growth of healthy microorganisms in intestinal environment, which display immunomodulatory functions, and inhibit the adherence and invasion of pathogens in the colonic epithelia (From Delgado *et al.* 2011). 26

CHAPTER 2

Figure 2.1. Sucrose hydrolysis catalyzed by FFase. 42

Figure 2.2. FOS production from sucrose by transfructosylation reaction catalyzed by FTase or FFase (adapted from Tokunaga 2004). 43

Figure 2.3. Detection of glucose (b), fructose (d) and glucose and fructose (f) in *A. pullullans* (standard microorganism) colonies grown in CD plates. Plates (a), (c) and (e) are the negative controls for each dye system. 46

Figure 2.4. Screening results of relative transfructosylation activity in *A. pullulans* (standard microorganism) (a), *A. ibericus* – MUM 03.49 (b) and *A. niger* – MUM 05.14 (b). 47

CHAPTER 3

Figure 3.1. Response surface described by the model in the region explored experimentally. 61

Figure 3.2. Comparison between experimental results and the values predicted by the model. 62

Figure 3.3. Kinetics of cell growth (cell growth/g.L^{-1}), total FOS production and sucrose consumption (concentration/g.L^{-1}) for *A. pullulans* using the optimized conditions. 63

LIST OF FIGURES (CONT.)

Figure 3.4. HPLC chromatogram of the fermentation medium in the FOS maximum yield production point (64,1 ± 0,0 $g_{FOS}/g_{sucrose}$) at the optimized fermentation conditions (temperature = 32 ºC, speed of agitation = 385 rpm). 63

CHAPTER 4

Figure 4.1. Schematic representation of the enzymatic extraction procedure of the different cellular spaces (extracellular, periplasmic and intracellular spaces) of *A. pullulans*. 75

Figure 4.2. Microscope images of *A. pullulans* cells after enzyme extraction procedures with 100 x magnification (I – cells on the culture broth; II – cells after treatment with Lyticase; III – cells disrupted by sonication). 77

CHAPTER 5

Figure 5.1. Fed-batch fermentation of sucrose by *A. pullulans* at constant feeding profile: kinetics of sucrose consumption and glucose, fructose, kestose, nystose and fructofuranosyl nystose production (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method. 89

Figure 5.2. Fed-batch fermentation of sucrose by *A. pullulans* at constant feeding profile: kinetics of cell growth (biomass concentration/g.L^{-1}), total FOS production and sucrose consumption (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method. 89

Figure 5.3. Fed-batch fermentation of sucrose by *A. pullulans* at constant feeding profile: Kinetics of total FOS production, sucrose consumption (concentration/g.L^{-1}) and transfructosylation and hydrolyzing activity (activity/U.mL^{-1}). The errors associated to sugar concentrations and enzymatic activity are the ones associated to the HPLC method. 90

Figure 5.4. Fed-batch fermentation of sucrose by *A. pullulans* at linear feeding profile: kinetics of sucrose consumption and glucose, fructose, kestose, nystose and fructofuranosyl nystose production (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method. 91

LIST OF FIGURES (CONT.)

Figure 5.5. Fed-batch fermentation of sucrose by *A. pullulans* at linear feeding profile: kinetics of cell growth (biomass concentration /g.L^{-1}), total FOS production and sucrose consumption (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method. 91

Figure 5.6. Fed-batch fermentation of sucrose by *A. pullulans* at linear feeding profile: kinetics of total FOS production, sucrose consumption (concentration/g.L^{-1}) and transfructosylation and hydrolyzing activity in the fermentation broth (activity/U.mL^{-1}). The errors associated to sugar concentrations and enzymatic activity are the ones associated to the HPLC method. 92

CHAPTER 6

Figure 6.1. Laboratory Pilot Fermenter from Bioengineering (Switzerland) used in this semi-pilot scale 75 L experiment. 100

Figure 6.2. Semi-pilot scale one-step batch fermentation of sucrose by *A. pullulans*: kinetics of sucrose consumption and glucose, fructose, kestose, nystose and fructofuranosyl nystose production (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method. 102

Figure 6.3. Semi-pilot scale one-step batch fermentation of sucrose by *A. pullulans*: kinetics of cell growth (cell growth/g.L^{-1}), total FOS production and sucrose consumption (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method. 102

Figure 6.4. Process flowsheet of FOS industrial production, implemented in SuperPro-Designer software. 104

LIST OF TABLES

CHAPTER 1

Table 1.1. Microbial sources of enzymes with transfructosylation activity – FOS producing enzymes. 18

Table 1. 2. Commercially produced food-grade FOS. 29

CHAPTER 2

Table 2.1. Fungi from MUM collection tested for relative transfructosylation activity. 45

Table 2.2. Screening results of relative transfructosylation activity in Fungi from MUM collection tested. 47

CHAPTER 3

Table 3.1. Experimental range and levels of the factors tested in the 22 full factorial central composite design. 56

Table 3.2. Experimental design of the 22 full factorial central composite design used for the optimization of the total FOS production. 56

Table 3.3. Experimental design and results of the 22 full factorial central composite design used for the optimization of the total FOS production. 59

Table 3. 4. Analysis of variance (ANOVA) for the second-order model determined for % total FOS (w/w). 60

Table 3.5. Regression analysis using the 22 full factorial central composite design. 60

Table 3.6. FOS yields obtained using FTase and/or FFase from various fungal strains. 65

LIST OF TABLES (CONT.)

CHAPTER 4

Table 4.1. Enzymatic activity (hydrolytic activity - U_h and transfructosylation activity - U_t) and total protein of the enzymes extracted from the extracellular, periplasmatic and intracellular spaces of *A. pullulans*. 78

CHAPTER 5

Table 5.1. FOS production yield (Y_P,% (g_{FOS}.gsucrose^{-1})) and productivity (Q, g.L^{-1}.h^{-1}) in “Fed-Batch 1” – constant feeding profile, “Fed-Batch 2” – linear feeding profile and Batch optimized experiment (data from Chapter 3). 90

CHAPTER 6

Table 6.1. Process economics (investment, operational cost, FOS net price, NPV, IRR and ROI) for a FOS production industry of 10.000 ton/year, based on a one-stage FOS production process. 105

LIST OF SYMBOLS

Uppercase Latin Letters

F	Feeding rate	$(L^{-3}t^{-1})$
F-value	F-test statistical parameter	
P_f	End FOS concentration measured in the aqueous phase	(ML^{-3})
P_i	Initial FOS concentration measured in the aqueous phase	(ML^{-3})
P-value	Statistical parameter	
Q	Process productivity	$(ML^{-3}t^{-1})$
Q^2	Prediction determination coefficient	
R^2	Determination coefficient	
$R^2_{adjusted}$	Adjusted determination coefficient	
S_0	Sucrose concentration of the feeding mash	(ML^{-3})
S_f	End sucrose concentration measured in the aqueous phase	(ML^{-3})
S_i	Initial sucrose concentration measured in the aqueous phase	(ML^{-3})
T	Temperature	(θ)
U_h	Hydrolytic activity	$(Nt^{-1}L^{-3})$
U_t	Transfructosylation activity	$(Nt^{-1}L^{-3})$
V_f	End volume of the fermentation medium	(L^3)
V_i	Initial volume of the fermentation medium	(L^3)
X_0	Real value of the independent factor at the central point	
X_i	Real value of the independent factor	
Y_P	Yield of FOS production per substrate consumption	(MM^{-1})

Lower Latin Letters

t_f	Fermentation time	(t)
t-test	Statistical parameter	
x_i	Coded value of the independent factor	
y	Optimal total FOS yield point	(ML^{-3})

Greek Letters

β	Parameters whose values are to be determined using multiple linear regression model	
ε	Random error term	
θ	Duration of the reactor feeding phase	(t)

Abbreviations

ANOVA	Analysis of variance
CD	Czapek dox media
CIT	Corporate income tax
CPB	Citric phosphate buffer
DERRAMA	Municipal tax
FAO	Food and agriculture organization
FDA	Food and drug administration
FDH	Fructose dehydrogenase
FFase	β-Fructofuranosidase
FOS	Fructooligosaccharides

FOSHU	Foods for specified health use
FTase	Fructosyl transferase
FUFOSE	Functional food science in Europe
GALT	Gut associated lymphoid tissue
GF2	1-Kestose
GF3	Nystose
GF4	1^F-β- Fructofuranosyl nystose
GOD	Glucose oxidase
GOS	Galactooligosaccharides
HPLC	High-performance liquid chromatography
IRR	Internal rate of return
MRLM	Multiple linear regression model
MUM	Micoteca da Universidade do Minho
NDO	Non-digestible oligosaccharides
NLEA	Nutrition labeling and education act
NPV	Net present value
OTA	Ochratoxin A
POD	Peroxidase
ROI	Return on investment
RSM	Response surface methodology
SCFAs	Short chain fatty acids
SmF	Submerged fermentation
SSF	Solid state fermentation

GENERAL INTRODUCTION



"If you can't explain it simply, you don't understand it well enough."

Albert Einstein.

This thesis addresses the development and optimization of a fermentation process for the production of fructooligosaccharides – FOS – by fungi. Conventionally, FOS production is a two-stage process that requires an enzyme production and purification step in order to proceed with the enzymatic reaction on its own. FOS production by fungi in bioreactors in a one-stage process constitutes an emergent field of opportunities as it reduces both investment and operation costs by avoiding the purification step of the FOS-producing enzyme from the cell extract. The use of the whole cell as the biocatalyst leads to a more competitive and less expensive process.

CONTEXT AND MOTIVATION

RESEARCH AIMS

OUTLINE OF THE THESIS

REFERENCES

CONTEXT AND MOTIVATION

Through much of its history, nutrition has concerned itself with the observation that deficiencies in the diet lead to disease states, and that deficiency diseases can be avoided by ensuring an adequate intake of the relevant dietary components. While food has long been used to improve health, the knowledge of health is now being used to improve food. Strictly speaking, all food is functional, in that it provides energy and nutrients necessary for survival. However, the term "functional food" in use today conveys health benefits that extend far beyond mere survival. Food and nutrition science has moved from identifying and correcting nutritional deficiencies to designing foods that promote optimal health and reduce the risk of disease (Clydesdale 2004, Howlett 2008).

In nutritional sciences there is much interest in dietary modulation of the human gut. The gastrointestinal tract, particularly the colon, is very heavily populated with bacteria. Although most bacteria are benign, certain gut species are pathogenic and may be involved in the onset of acute and chronic disorders. *Bifidobacterium* sp. and *Lactobacillus* sp., the most common probiotics, are thought to be beneficial and are common targets for dietary intervention. Prebiotic is a non-viable food ingredient selectively metabolized by beneficial intestinal bacteria, modulating the gut microbiota and improving health by stimulating numbers and/or activities of *Bifidobacterium* sp. and *Lactobacillus* sp. An 'optimal' gut microbiota can increase resistance to pathogenic bacteria, lower blood ammonia, increase stimulation of the immune response and reduce the risk of cancer (Manning and Gibson 2004, Saad *et al.* 2013).

Fructooligosaccharides (FOS), lactulose and galactoligosaccharides (GOS) are all popular prebiotics. In Europe, FOS have been the most commonly used and successful prebiotics. They are used as components of functional foods, that have received the GRAS status (generally recognized as safe) from the FDA (Food and Drug Administration—U.S.), and are worth about 150 €/kg (US $200/kg). Due to their nutrition- and health-relevant properties, like moderate sweetness (typically 30 % – 60 % of the sucrose value), low cariogenicity, a low calorimetric value, and a low glycemic index, they have been (since 1980) increasingly used by the food industry (beverages, sweets) for modifying viscosity, emulsification capacity, gel formation, freezing point, and colour of foods (Maiorano *et al.* 2008, Saad *et al.* 2013).

In the last two decades, several studies were developed on the production of FOS as food ingredients. In industry, FOS are typically produced from the disaccharide sucrose using enzymes with transfructosylation activity using a two-stage process. In the first step, enzymes with transfructosylation activity (β-fructofuranosidase fructohydrolase, FFase, EC 3.2.1.26 and sucrose fructosyl transferase, FTase, EC 2.4.1.9) are produced by microorganisms, usually fungi (e.g. *Aspergillus* sp., *Aureobasidium* sp., *Penicillium* sp.) and purified from the fermentation broth. In the second step, FOS are synthesized from sucrose by FFase and/or FTase produced in the first

stage of the process, being glucose and fructose obtained as reaction by-products (Sangheeta *et al.* 2005, Dominguez *et al.* 2012).

The yields of transfructosylation reactions reported are usually between 54 % – 60 % (Sangeetha *et al.* 2005, Mussato *et al.* 2012) and the development of a process more economically attractive for FOS production at an industrial level is highly desirable. Although many studies have been carried out towards this purpose, up to date no relevant results capable of replacing the current industrial process were reported, and the awareness of new methods to improve FOS yield with lower cost production, as well as additional FOS benefits, are still a big challenge and will remain an interesting research field in the coming years.

RESEARCH AIMS

The main purpose of this thesis was to develop and optimize a one-stage fermentation process for a more competitive and less expensive fructooligosaccharides (FOS) production towards its industrial implementation. The specific aims to accomplish the main purpose were:

- to screen fungi in order to select strains that demonstrate higher enzymatic activity as FOS-producing microorganisms;
- to develop and optimize a one-stage fermentation process for FOS production with higher production yield, with the purpose of eliminating the enzyme separation and purification steps (characteristic steps of the conventional production process);
- to determine the suitability and economic viability of the optimized FOS production process at a semi-pilot scale.

OUTLINE OF THE THESIS

In order to cover the abovementioned research aims, this thesis is organized in six chapters. The thesis aims and subjects are covered in this chapter, and in Chapter 7 the main conclusions and recommendations from the current work are presented. In the other chapters, the research findings of this work are covered as follows:

- In Chapter 1, the concepts of functional food, prebiotic, probiotic and symbiotic are reviewed. The role of FOS as a prebiotic with functional properties is presented. Emphasis is given to FOS occurrence, production and market data.

- In Chapter 2, the development of a presumptive and indirect colorimetric plate assay for the evaluation of transfructosylation activity in fungi is described. Screening of more than 40 fungal strains from Micoteca da Universidade do Minho (MUM) collection with the developed method is presented.

- In Chapter 3, the optimization by experimental factorial design and response surface analysis of the fermentation conditions, using an integrated one-stage method, for maximization of FOS production via sucrose fermentation by *Aureobasidium pullulans*, is described. The optimized yield of FOS production and productivity are presented.

- Transfructosylating and hydrolyzing activities of the enzymes extracted from the different cellular spaces (extracellular, periplasmic and intracellular) of *A. pullulans* are presented in Chapter 4. The enzyme extraction procedures optimized for fungi species are also presented.

- In Chapter 5, FOS production by fed-batch fermentation of sucrose by *A. pullulans* with two feeding profiles is described. FOS production yield and productivity of fed-batch experiments are determined and compared to the ones obtained in the batch culture.

- In Chapter 6, scale-up of the one-stage optimized process for FOS production by sucrose fermentation by *A. pullulans* (developed in Chapter 3) is presented. The effect of scaling the process 25 times is evaluated. A rough economical analysis for FOS industrial production by one-stage process is given and compared to the current two-stage industrial process.

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CHAPTER 1:
FRUCTOOLIGOSACCHARIDES: INGREDIENTS FOR FUNCTIONAL FOOD



"Let food be thy medicine and medicine be thy food"

Hippocrates.

In this chapter, some important concepts such as functional food, prebiotic, probiotic and synbiotic are briefly overviewed. A special emphasis is given to Fructooligosaccharides (FOS), as a prebiotic, being presented their characteristics, occurrence, production, functional properties and market data.

1.1. INTRODUCTION

1.2. FUNCTIONAL FOODS

1.3. PROBIOTICS

1.4. PREBIOTICS

1.5. SYNBIOTICS

1.6. FRUCTOOLIGOSACCHARIDES AS PREBIOTICS

1.6.1. FOS occurrence

1.6.2. FOS production

1.6.2.1. FOS microbial production – transfructosylation enzymes

1.6.2.2. FOS production by submerged fermentation (SmF)

1.6.2.3. FOS production by solid state fermentation (SSF)

1.6.2.4. Production of high-content FOS

1.6.3. FOS functional properties

1.6.4. FOS market data

1.7. CONCLUSIONS

1.8. REFERENCES

1.**1. INTRODUCTION**

Through much of its history, nutrition has concerned itself with the observation that deficiencies in the diet lead to disease states and that deficiency diseases can be avoided by ensuring an adequate intake of the relevant dietary components (Howlett 2008).

While food has long been used to improve health, the knowledge of health is now being used to improve food. The first systematic exploration of the positive aspects of food functionality was undertaken in Japan were research programmes funded by the Japanese government during the 1980s focused on the ability of some foods to influence physiological functions (Clydesdale 2004, Howlett 2008).

Strictly speaking, all food is functional, in that it provides energy and nutrients necessary for survival. But, the term “functional food” in use today conveys health benefits that extend far beyond mere survival. Food and nutrition science has moved from identifying and correcting nutritional deficiencies to designing foods that promote optimal health and reduce the risk of disease (Clydesdale 2004).

On the road to optimum (optimized) nutrition, which is an ambitious and long-term objective, functional food is, among others, a new, interesting and stimulating concept, as much as it is supported by sound and consensual scientific data generated by the recently developed functional food science aimed at improving dietary guidelines by integrating new knowledge on the interactions between food components and body functions and/or pathological processes (Roberfroid 2000a).

The gut is an obvious target for the development of functional foods, because it acts as an interface between the diet and all other body functions. Over the past decade, we have witnessed an explosion of new information pertaining to the possible role of gut microbiota in health and disease. This has led to an interest in the development of strategies aimed at dietary modulation of the microbiota composition and activity, achieved by the use of pre-, pro- and a combination of both (synbiotics) (Howlett 2008, Szajewska 2010, De Preter *et al.* 2011).

Various non-digestible carbohydrates have been tested for their effect on the microbial community. Among them, the functional oligosaccharides present important physicochemical and physiological properties beneficial to the consumer’s health, and therefore their use as food ingredients has increased rapidly. The functional oligosaccharides are intermediate in nature between simple sugars and polysaccharides and are claimed to behave as dietary fibres and prebiotics (Simmering and Blaut 2000, Kunz and Rudloff 2006, Mussato and Mancilha 2007).

Fructooligosaccharides (FOS), galactooligosaccharides (GOS), isomaltooligosaccharides, soybean oligosaccharides, xylooligosaccharides and maltitol are among the commonly used

prebiotics, being FOS the most studied and the best implemented oligosaccharides in the European market (Chen *et al.* 2000, Qiang *et al.* 2009, Nobre *et al.* 2012).

1.2. FUNCTIONAL FOODS

As a working definition, a food can be said to be functional if it contains a component (whether or not a nutrient) that benefits one or a limited number of functions in the body in a targeted way that is relevant to either the state of well-being and health or the reduction of the risk of a disease, or if it has psychological effect beyond the traditional nutritional effect (Roberfroid 2000b).

Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern. A functional food can be a natural food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means. It can also be a food where the nature of one or more components has been modified, or a food in which the bioavailability of one or more components has been modified, or any combination of these possibilities. It also might be functional for all members of a population or for particular groups of the population, which might be defined, for example, by age or by genetic constitution (Diplock *et al.* 1999).

Research on functional foods began in Japan in the early 1980s, when 86 specified research programs on “systematic analysis and development of food functions” were funded by a scientific fellowship grant from the Ministry of Education. This led, in 1991, to the definition in Japanese law of a category of “foods for special dietary use”, which were allowed to carry claims for specific health effects on their labeling. The claims had to be substantiated in order to receive the approval of the Japanese Ministry of Health and Welfare, and the foods could then be designated as “Foods for Specified Health Use” (FOSHU). In the United States, the Nutrition Labeling and Education Act (NLEA), which was established in 1990 and first enforced completely in 1994, allows health claims to be made for foods containing ingredients for which the Food and Drug Administration (FDA) has scientific evidence demonstrating a correlation between intake and prevention or cure of certain diseases. In the latter half of the 1990s, the European Commission funded an activity to establish a science-based approach to exploring the concept of functional foods. This Concerted Action, “Functional Food Science in Europe” (FUFOSE), involved a large number of European experts in nutrition and related sciences and produced a consensus report that has become widely used as a basis for discussion and further evolution of thinking on the topic. FUFOSE developed a working definition of a functional food as one that is “satisfactorily

demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and wellbeing and/or reduction of risk of disease". In the context of this working definition, a "target function" is a biological activity ongoing in the body that is a target for intervention with a view to the maintenance or improvement of health and well-being and/or reduction of risk of disease (Diplock *et al.* 1999, Roberfroid 2000b, Howlett 2008).

The global market for functional food is in fast growth since the early 2000's, and in 2008 the entire functional foods market was worth over an estimated 80 billion US$, with United States holding the major share in the nutraceuticals market (35 %) followed by Japan (25 %) and together with the ever-growing European market (current estimate 8 billion US$). India and China are the two major countries known for their production of traditional functional food products and nutraceuticals, but other South-East Asian countries and Gulf nations are developing potential markets (Vergari *et al.* 2010). In the United States, functional foods have a share of about 2-3 % of the food market, and this percentage doubled by 2008 (Siró *et al.* 2008). On the other hand in Japan, that is considered the birthplace of functional foods, the market for these foods is very important. Indeed, more than 1700 products were launched on the market between 1988 and 1998, with a turnover estimated of around 14 billion US$ in 1999, being in 2005, more than 500 products labeled as FOSHU (Menrad 2003, Saad *et al.* 2013). As for the European market, it has been estimated between 4 and 8 billion US$ in 2003 and this value increased to about 15 billion US$ in 2006, with a market share of around 1 % of food market. Germany, France, United Kingdom and the Netherlands represent the most important countries within the functional food market in Europe (Mäkinen-Aakula 2006, Saad *et al.* 2013). However, it should be emphasized that the European functional food market is very heterogeneous. In general, the interest of consumers in functional food in the Central and Northern European countries is higher than in Mediterranean countries, where consumers appreciate natural, fresh foods and consider them better for health (van Trijp and van der Lans, 2007). The functional food regulatory situation in Europe is also a challenge to industry. From a legal point of view, functional food is positioned in a transitional zone between food and pharmaceuticals. In almost all European countries, as well as in the European Union, these areas are traditionally regulated by separate institutions and are subject to different regulations, creating a kind of "grey zone" with a high level of uncertainty (Siró *et al.* 2010, Vergari *et al.* 2010).

Evidence on the impact of the gut microbial activity on human health is rapidly expanding and several host–microbiota interactions, both positive and negative, have been reported. The human large intestine is recognized as one of the most metabolically active organs of the human body and the resident microbial ecosystem is extremely complex and dynamic with high densities of living bacteria (Howlett 2008, De Preter *et al.* 2011). It exerts a considerable influence on host biochemistry including:

- enzymatic activity of intestinal contents;
- short chain fatty acid (SCFA) production in the lumen
- oxidation-reduction potential of luminal contents;
- host physiology;
- modification of host-synthesized molecules.

The gut also provides the basis for a barrier that prevents harmful bacteria from invading the gastrointestinal tract. Moreover it plays a major role in eliciting, at an early age, an immune system which has a measured response to foreign proteins as potential antigens, balanced with an effective resistance to infection. An interesting aspect is the possibility of therapeutically manipulating the intestinal microbiota to improve the host's health by administering several microorganism species such as bifidobacteria, collectively named probiotics. Prebiotics, such as FOS, promote the growth of bifidobacteria, potentiating their effects. The combination between a probiotic and a prebiotic is known as a synbiotic. Thus, this is one of the most promising areas for the development of functional food components since it relies in the use of probiotics, prebiotics and synbiotics to modify the composition and the metabolic activity of the gut microbiota (Ziemer and Gibson 1998, Simmering and Blaut 2001, Howlett 2008, Ribeiro 2011).

1.3. PROBIOTICS

The human gastrointestinal tract contains a complex ecosystem of microorganisms, including bacteria, fungi and the Archaea. These species are termed "normal flora", or "microbiota". It is estimated that 500 to 1.000 different species of bacteria live in the human body. The role of the resident microbiota in the overall health and well-being of humans is now recognized as crucial. This community has profound effects on the daily welfare of the host, providing energy, nutrients, bioactive compounds, detoxification, and resistance to infectious diseases. Common bacterial species found in the small intestine include *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Mycobacterium*, *Enterococci* and *Enterobacteriaceae* (Reid *et al*. 2003, Sears 2005, Jacobs *et al*. 2007, Ndagijimana *et al*. 2009, Serino *et al*. 2009).

Probiotics have a long history of use. In fact, the first records of intake of bacterial drinks by humans are over 2000 years old. Probiotics are widely used to prepare fermented dairy products such as yogurt or freeze-dried cultures, but in the future they may also be found in fermented

vegetables and meats. According to the current definition of the United Nations' Food and Agriculture Organization (FAO), probiotics are live microorganisms which, when administrated in adequate amounts confer a health benefit on the host. When used in food, probiotic organisms must be able to survive the passage through the gut; i.e. they must be able to resist gastric juices and exposure to bile. Furthermore, they must be able to proliferate and colonize the digestive tract. In addition, probiotics must be safe and effective, and also maintain their effectiveness and potency for the duration of the shelf-life of the product. The most common probiotic bacteria presently used in food products belong to the genera *Bifidobacterium* and *Lactobacillus*, both of which form part of the normal intestinal microbiota. Under natural conditions a protective gut microbiota develops and there is no need for a probiotic supplement; but humans live under rather unnatural conditions as we eat a great deal of processed and in many cases sterile food which may affect our access to, and colonization by, certain types of bacteria. Humans also consume antibacterial substances ranging from vinegar to antibiotics (Fuller 1991, Roberfroid 2000c, Simmering and Blaut 2001, Gibson 2004, Saad *et al*. 2013), thus resulting in an imbalance of the gut microbiota.

A probiotic microorganism should be of human origin, demonstrate non-pathogenic behavior, exhibit resistance to technological processes, prove resistant to gastric acid and bile, adhere to gut epithelial tissue, be able to persist, albeit for short periods, in the gastrointestinal tract, produce anti-microbial substances, modulate immune responses and have the ability to influence metabolic activities (e.g. cholesterol assimilation, lactase activity, vitamin production).

Probiotics enrich the population of gut microbiota species that preferentially ferment carbohydrates and have little proteolytic activity, and several health-related effects associated with their intake, including alleviation of lactose intolerance, immune enhancement and hypocholesterolemic effect, have been reported in human studies. Also rotavirus-induced diarrhea and possibly colon cancer are disorders for which there is evidence of disease reduction from probiotic consumption (Roberfroid 2000c, De Preter *et al*. 2011). An effective way to fortify probiotic activity in the gut is to assay fermentation characteristics and attempt to selectively stimulate probiotics through increasing their metabolic capacity, which lead us to the prebiotic concept. (Gibson 2004).

1.4. PREBIOTICS

A prebiotic was originally defined in 1995 as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid 1995). A more

recent definition stated that “a prebiotic is a selectively fermented ingredient that allows specific changes; both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health” (Gibson *et al.* 2004). The principal concept associated with both definitions is that the prebiotic has a selective effect on the microbiota that results in an improvement in the host health. According to FAO, these definitions of prebiotic, while differentiating this class of non-digestible food ingredient within the dietary fibres and broadly serving the more common and well studied prebiotic oligosaccharides, is restrictive in its applicability for target sites outside the gastrointestinal tract. Thus, a new prebiotic definition was proposed by FAO: “a prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota” (Piñeiro *et al.* 2008).

Prebiotics represent a second strategy to improve the balance of intestinal bacteria. Rather than introducing exogenous strains into an individual’s intestinal tract, prebiotics aim to selectively stimulate the proliferation and/or activity of advantageous groups of bacteria already present in the intestinal microbiota. As a consequence, prebiotics can constitute a more practical and efficient way to manipulate the gut microbiota than probiotics. However, the other side of the coin is that if for any reason, such as disease, ageing, antibiotic or drug therapy, the appropriate health-promoting species are not present in the bowel, the prebiotic is unlikely to be effective (Playne and Crittenden 2004, Macfarlene *et al*. 2008).

The gut microbiota ferments a range of substances, mainly provided by the diet, which cannot be digested by the host in the small intestine and are available for fermentation by the colonic microbiota. These include resistant starch, non-starch polysaccharides (dietary fiber), oligosaccharides, proteins, amino acids, among others. In a typical adult, about 100 g of food ingested each day reaches the large intestine and is therefore susceptible to fermentation by the gut microbiota. Key criteria for a food ingredient to be classified as a prebiotic are (i) it must not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract so that it reaches the colon in significant amounts, and (ii) it must be a selective substrate for one or more beneficial bacteria that are stimulated to grow. It may also induce local (in the colon) or systemic effects through bacterial fermentation products that are beneficial to host health (Manning and Gibson, 2004, Howlett 2008, De Preter *et al*. 2011).

These ingredients are normally restricted to certain carbohydrates (particularly oligosaccharides) but could include proteins, peptides and lipids. Oligosaccharides are defined as glycosides composed of between 3 and 10 monomer sugar units. Non-digestible oligosaccharides (NDO) can be distinguished from other carbohydrates on the basis of being resistant to digestion in the stomach and small intestine (Playne and Crittenden 2004).

The concept of prebiotics arose from the observation that inulin and FOS selectively stimulate the growth of bifidobacteria which are considered to be beneficial for human health. The majority of studies have so far focused on inulin, FOS and GOS. These saccharides have now a long history

of safe use and are generally regarded as safe (Gibson *et al*. 2004, Macfarlene *et al*. 2006, Piñeiro *et al*. 2008).

Most prebiotics and prebiotic candidates currently identified are NDO and are obtained either by extraction from plants (e.g., chicory inulin), possibly followed by an enzymatic hydrolysis (e.g., oligofructose from inulin) or by synthesis (by transglycosylation reactions) from mono- or disaccharides such as sucrose (FOS) or lactose (GOS) (Crittenden and Playne, 1996). Nevertheless, other NDO including xylooligosaccharides, soybean oligosaccharides and isomaltooligosaccharides have also been tested for their prebiotic effect (Gibson and Roberfroid 1995, Simmering and Blaut 2001).

Apart from their potential to modify the gut microbiota and its metabolic activities in a beneficial manner, many other helpful effects of prebiotics are being investigated. These include their ability to modulate gut function and transit time, to activate the immune system, to increase the production of butyric and other short-chain fatty acids, to increase the absorption of minerals, such as calcium and magnesium, and to inhibit lesions that are precursors of adenomas and carcinomas. Thus, they could have the potential to help reduce some of the risk factors involved in the causes of colorectal diseases and reduce the risk of civilization diseases such as cardiovascular disease, colon cancer and obesity. Strategies for developing prebiotic products as functional foods aim to provide specific fermentable substrates for beneficial bacteria (bifidobacteria and lactobacilli). These may provide beneficial amounts and proportions of fermentation products, especially in the lower part of the colon where the effects are believed to be most favorable. Although the association with past outbreaks will remain conjectural, it is reasonable to suggest that prebiotic administration may possible exert very important prophylactic effects directed towards gut pathogens. Even as part of a more complex story, the potential of prebiotics is too large to be ignored (Ziemer and Gibson 1998, Howlett 2008, Quiang *et al*. 2009, Shimizu and Hachimura 2011).

1.5. SYNBIOTICS

A synbiotic has been defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare. They can enhance health promotion in a synergistic manner, over either probiotics or prebiotics alone. One of the principal benefits of synbiotics is believed to be the increased persistence of the probiotic in the gastrointestinal tract. The live microbial additions (probiotics) may be used in conjunction with

specific substrates (prebiotics) for growth (e.g. a fructooligosaccharide in conjunction with a bifidobacteria strain or lactitol in conjunction with a lactobacillus organism). Survival, colonization and the beneficial effects of feeding an exogenous probiotic may be enhanced and extended by simultaneous administration of a prebiotic which can be used by the probiotic within the intestinal tract (Gibson and Roberfroid 1995, Collins and Gibson 1999, Rastall and Maitin 2002, De Preter *et al.* 2011, Rodrigues *et al.* 2011).

The prebiotics used in synbiotic combinations are often currently selected on the basis of food technology issues and may not in all cases be the optimal complement for the probiotic strains. However, if synergy between the probiotic and prebiotic ingredients is to be exploited, the probiotic strain should be able to utilize the prebiotic as a carbon substrate in the gut efficiently. (Playne and Crittenden 2004).

1.6. FRUCTOOLIGOSACCHARIDES AS PREBIOTICS

Of all NDO known so far, FOS have attracted special attention. FOS is a common name for fructose oligomers and are usually understood as inulin-type oligosaccharides. They constitute a series of homologous oligosaccharides derived from sucrose usually represented by the formula GF_n, that are mainly composed of 1-kestose (GF2), nystose (GF3), and 1^F-β- fructofuranosyl nystose (GF4), in which two, three, and four fructosyl units are bound at the β-2,1 position of glucose, respectively, as shown in Figure 1.1. (Yun 1996, Lee *et al.* 1999).

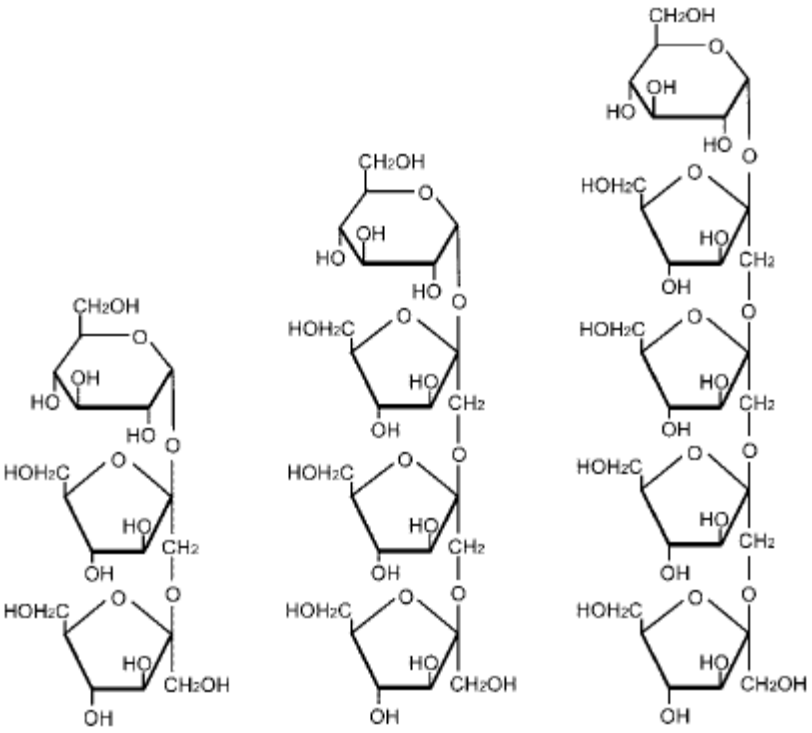


Figure 1.1. Structures of 1-kestose (GF2) (left), nystose (GF3) (center), and fructofuranosyl nystose (GF4) (right).

1.6.1. FOS occurrence

Fructooligosaccharides (FOS) are found in trace amounts as natural components in fruits, vegetables and honey. As a series of fructose oligomers and polymers derived from sucrose, they occur in many higher plants as reserve carbohydrates. Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato and rye are special sources of FOS (Yun 1996, Sangeetha *et al.* 2005a, Mussato and Mancilha 2007).

For most of these sources, concentrations range between 0,3 % and 6 % of fresh weight; for chicory these values are between 5 % and 10 %, while in Jerusalem artichoke they can go up to 20 %. Based on the average consumption of these natural sources, an average daily consumption of FOS of approximately 13,7 mg/kg body weight/day or 806 mg/day has been estimated (Voragen 1998).

1.6.2. FOS production

FOS represent one of the major classes of bifidogenic oligosaccharides in terms of their production volume. They can be produced by hydrolysis of inulin or by transfructosylation of sucrose. The FOS mixture obtained by enzymatic hydrolysis of inulin closely resembles the mixture produced by the transfructosylation process, although slightly different end products are obtained as not all of the β (1→2)-linked fructosyl chains end with a terminal glucose. Additionally, the oligosaccharide mixture produced from inulin hydrolysis contains longer fructo-oligomer chains than that produced by the sucrose transfructosylation process. In this process, FOS are produced from the disaccharide sucrose using enzymes with transfructosylation activity. These enzymes are invertase, (β-fructofuranosidase fructohydrolase, FFase, EC 3.2.1.26) and sucrase (sucrose fructosyl transferase, FTase, EC 2.4.1.9), which are mainly derived from fungal and bacterial sources. A high concentration of the starting substrate is required for efficient transfructosylation. FOS, namely kestose, nystose and fructofuranosyl nystose (Figure 1.1) are formed, as well as by-products of the reaction such as glucose and small amounts of fructose (Brenda 2005, Crittenden and Playne, 1996, Singh and Singh 2010, Saad *et al.* 2013), being this process thoroughly discussed in the next section.

1.6.2.1. FOS microbial production – transfructosylation enzymes

FOS are produced commercially through the enzymatic synthesis from sucrose by microbial enzymes with transfructosylation activity. Several bacterial strains have been reported to produce enzymes with transfructosylation activity. Nevertheless, FOS producing enzymes are rare in bacteria. On the other hand, several fungal strains, such as *Aspergillus* sp., *Aureobasidium* sp. and *Penicillium* sp., are known to produce extracellular and/or intracellular enzymes with transfructosylation activity (Table 1.1).

Table 1.1. Microbial sources of enzymes with transfructosylation activity – FOS producing enzymes.

Source	Microorganism	Enzyme	Reference
Fungal transfructosylation enzyme	*Aureobasidium pullulans*	Intra and extracellular FTase	Yun *et al.* 1997,
		Intra and extracellular FTase	Sangeetha *et al*. 2004a,
		Intra and extracellular FTase	Vandáková *et al*. 2004,
		Intracellular FTase	Lateef *et al*. 2007
		Periplasmic FFase	Yoshikawa *et al*. 2006
		Extracellular FTase	Dominguez *et al*. 2012
	Aspergillus aculeatus	FTase from commercial enzyme preparation – Pectinex Ultra SP-L	Nemukula *et al*. 2009
			Ghazi *et al*. 2005
			Ghazi *et al*. 2007
	Aspergillus japonicus	Intra and/or extracellular FFase	Chen and Liu 1996
		Intra and/or extracellular FFase	Wang and Zhou 2006
		Extracellular FFase	Mussato *et al*. 2009
		Extracellular FFase	Mussato and Teixeira 2010
	Aspergillus niger	Extracellular FFase	Wallis *et al*. 1997
		Intra and extracellular FFase	Nguyen *et al*. 1999
		Intra and/or extracellular FTase	L'Hocine *et al*. 2000
	Aspergillus oryzae	Extracellular FTase	Sangeetha *et al*. 2004a,
		Extracellular FTase	Sangettha *et al*. 2005a
	Penicillium citrinum	Intra and/or extracellular enzyme	Lim *et al*. 2005
Bacterial transfructosylation enzyme	*Bacillus macerans*	Extracellular transfructosylation enzyme	Park *et al*. 2001
	Lactobacillus reuteri	Intra and/or extracellular FTase	van Hijum *et al*. 2002
	Zymomonas mobilis	Extracellular levansucrase	Bekers *et al*. 2002

Purification and characterization of an enzyme is a necessary step to aid the understanding of its mode of action. Many authors have reported the purification and characterization of FOS producing enzymes from various sources and by different microorganisms (bacteria and fungi) (Hayashi *et al.* 1991, L'Hocine *et al.* 2000, Park *et al.* 2001, Nguyen *et al.* 2005, Jedrzejczak-Krzepkowska *et al.* 2011). Although these proteins differ in their subunit structure, molecular weight, degree of glycosylation, chemical susceptibility and substrate specificity, they all display both hydrolytic and transfer activities. The same microorganism can even produce several enzymes with transfructosylation activity holding different characteristics (Yoshikawa *et al.* 2007). Fungal FTases have molecular masses ranging between 180.000 and 600.000, and are homopolymers with 2 to 6 monomer units. Some studies stated the optimum temperature and pH for these enzymes activity between 50 ºC to 60 ºC and 4,5 to 6,5, respectively (Madlová *et al.* 2000, Maiorano *et al.* 2008).

There are mainly two methods that can be used to produce transfructosylation enzymes and FOS by fermentation, namely by submerged fermentation (SmF) or by solid state fermentation (SSF). These two methods will be further discussed in the following sections.

1.6.2.2. FOS production by submerged fermentation (SmF)

The most common and studied method to produce FOS is by transfructosylation of sucrose, in a two stage process, schematically represented on Figure 1.2, being the enzyme(s) produced by SmF in the first stage. This is also the usual process used to produce the FOS that are currently available on the market (Singh and Singh 2010).

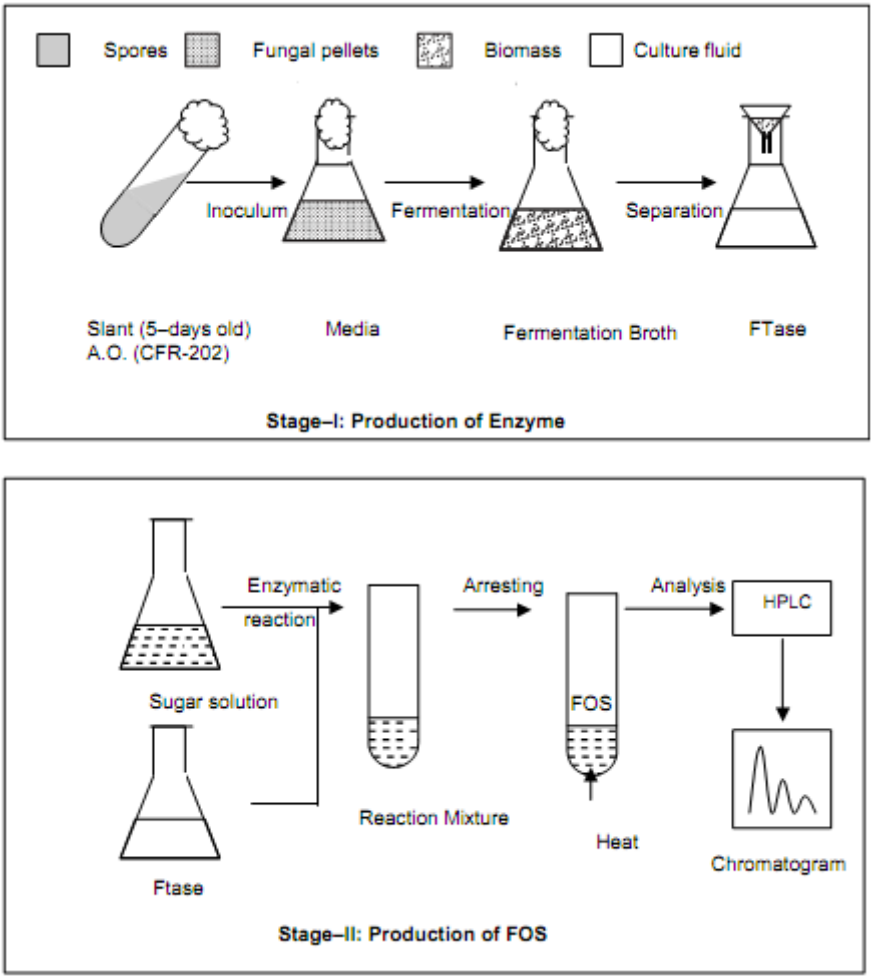


Figure 1.2. Schematic representation of the two stage FOS production process by SmF (From Sangeetha *et al*. 2005b).

Most studies on the experimental conditions for enzyme production are mainly based on shake-flask experiments, and the work carried out in bioreactors is not so usual. The variables usually studied to define the best operational conditions for enzyme production are the carbon and nitrogen sources, their concentrations, time of cultivation, agitation and aeration rates. Other factors also evaluated are related to the addition of different mineral salts, small amounts of amino acids, polymers and surfactants (Maiorano *et al.* 2008).

The production of transfructosylation enzymes is significantly influenced by the carbon source. Chen and Liu (1996) evaluated the effect of various carbon and nitrogen sources in enzyme production and found that sucrose at 25 % (w/v) and yeast extract were the best ones, respectively. Later on, Antošová *et al.* (2002) obtained the highest FTase activity using sucrose at 350 g/L and the authors found that biomass production was not influenced by sucrose concentration ranging from 50 g/L to 350 g/L. Vandáková *et al.* (2004) also studied the effect of sucrose on FTase production by *A. pullulans* and reported that sucrose content did not influence the total production of FTase. However, high sucrose concentrations resulted in high specific activities of the cells.

The effects of inorganic salts are reported by various authors. Chen and Liu (1996) studied the effect of inorganic salts and addition of $MgSO_4$. $7H_2O$. K_2HPO_4 was found to shift the morphology of the fungal growth from filamentous to pellet form without affecting FFase production. The morphology of fungal growth can range from dispersed mycelia to compact pellets. Control of mycelia morphology in fermentations is often a prerequisite for industrial applications. Although fungus, grown in the form of mycelia, may have a high specific growth rate, growth of fungus in the form of pellets is more attractive to many industrial fermentation processes from the perspective of reducing the power input needed for adequate gas dispersion and mixing (Metz and Kossen 1977, Chen and Liu 1996). K_2HPO_4 is described as a microelement source for cell growth, as well as a buffering reagent. Its optimal concentration ranges between 4 g/L (Sangeetha *et al*. 2005a, Sangeetha *et al*. 2005c) and 5 g/L (Vandáková *et al*. 2004, Shin *et al*. 2004, Lim *et al*. 2006). Mg^{2+} affects the permeability of the cell wall for *A. pullulans* (Vandáková *et al*. 2004) and its optimal concentration varies from 0,3 g/L (Sangeetha *et al*. 2005a, Sangeetha *et al*. 2005c) to 2 g/L (Balasubramaniem *et al*. 2001). $NaNO_3$ is the most common source of inorganic nitrogen in transfructosylation enzymes production by fungi and can be employed from 2 g/L (Lim *et al*. 2006) to 25 g/L (Dhake and Patil 2007). Others ions, such as $FeSO_4$ and $CuSO_4$, can also be added to the medium with positive effect (Balasubramaniem *et al*. 2001, Lim *et al*. 2006, Wang and Zhou 2006).

The effect of the medium pH in FTase production and microorganism growth has been reported. The pH of 5.5 was found to be the best initial value for FTase production by *A. oryzae* CFR202 (Sangeetha *et al*. 2005a, Sangeetha *et al*. 2005c), *A. japonicus* JN19 (Wang and Zhou 2006) and *P. purpurogenum* (Dhake and Patil 2007).

The FOS production yield by *A. pullulans*, in a two-stage process (being the first stage the transfructosylation enzyme production by SmF) can vary between 44,0 % ($g_{FOS}/g_{sucrose}$) (Shin *et al*. 2004) and 62,0 % ($g_{FOS}/g_{sucrose}$) (Yoshikawa *et al*. 2008). For *A. japonicus* these values can vary between 55,8 % ($g_{FOS}/g_{sucrose}$) (Wang and Zhou 2006) and 61,0 % ($g_{FOS}/g_{sucrose}$) (Chiang *et al*. 1997), whereas for *A. niger*, values ranging between 24,0 % ($g_{FOS}/g_{sucrose}$) (Nguyen *et al*. 1999) and 70,0 % ($g_{FOS}/g_{sucrose}$) (Maldová *et al*. 1999) can be expected. On the other hand, Sangeetha *et al*. (2005a, 2005c) reported a FOS production yield by *A. oryzae* of 53,0 % ($g_{FOS}/g_{sucrose}$) to 57,4 % ($g_{FOS}/g_{sucrose}$).

1.6.2.3. FOS production by solid state fermentation (SSF)

Despite most of all industrial enzymes are produced in SmF, SSF presents an interesting potential for small-scale units. Some advantages of this process are simplicity of operation, high volumetric productivity, product concentration and low capital cost and energy consumption. Moreover, the risk of contamination is reduced. SSF requires low water volume and thus has large impact on the economy of the process due to smaller fermenter-size, reduced downstream processing, reduced stirring and lower sterilization costs (Sangeetha *et al*. 2005b, Mussato and Teixeira 2010). However, there are also some disadvantages associated with the use of SSF, which have discouraged the use of this technique for industrial production. The main drawbacks are due to the build-up of gradients — of temperature, pH, moisture, substrate concentration or pO2— during cultivation, which are difficult to control under limited water availability, leading to scale-up engineering problems (Pandey 2003, Hölker *et al.* 2004).

The use of low cost agricultural and agro-industrial residues as substrates, contributes also to the lower capital and operating costs as compared to SmF. Several agricultural by-products like cereal bran, corn products, sugarcane bagasse and by-products of coffee and tea processing industries were used as substrates to produce FTase in SSF by *A. oryzae* CFR 202 (Sangeetha *et al.* 2004b). In this study, rice bran and wheat bran were found to be the substrates that maximize FTase production, with optimum activity at 60 ºC and pH 6.0. The maximum FOS production (52,0 % ($g_{FOS}/g_{sucrose}$)) was obtained after 8 h of reaction. Mussato *et al.* (2010) reported the increase in FOS yield and productivity by SSF with *A. japonicus* using agro-industrial residues as immobilization support and nutrient source. Corn cobs, coffee silverskin, and cork oak were used as support and nutrient source and the coffee silverskin was found to be the one that provided the higher production yield and FFase activity, reaching 70,0 % ($g_{FOS}/g_{sucrose}$) after 16 h of reaction. These authors (Mussatto *et al.* 2012) conducted a similar study with *P. expansum* where the use of low-cost materials including synthetic fiber, polyurethane foam, stainless steel sponge, loofah sponge, and cork oak were tested as carriers for the fungus immobilization.

Additionally, production of FOS and FFase by repeated batch fermentation of *P. expansum* immobilized on synthetic fiber was studied, reaching production yields of 87 % ($g_{FOS}/g_{sucrose}$), 72 % ($g_{FOS}/g_{sucrose}$) and 44 % ($g_{FOS}/g_{sucrose}$), in the 3 initial cycles (36 h, 48 h and 60 h, respectively).

Generally, the most efficient culture media and strain to produce enzymes in SSF are not the same as those in SmF, and vice versa (Maiorano *et al.* 2008). Optimization of fermentation medium for β-fructofuranosidase production by *A. niger* NRRL 330 in SSF and SmF was carried out using a fractional factorial design (Balasubramaniem *et al.* 2001). The results showed different compositions of optimized media for SmF and SSF. After optimization procedures the medium composition for SSF required twice more sucrose and yeast extract than the medium for SmF, and the productivity in SSF was about twice that in SmF.

1.6.2.4. Production of high-content FOS

Industrial production of FOS carried out with microbial transfructosylation enzymes was found to give a maximum theoretical yield of 55 % - 60%, based on the initial sucrose concentration (Sangeetha *et al.* 2005b). This yield cannot be increased due to the high amounts of glucose co-produced during the fermentation which acts as an inhibitor (Yun 1996).

Therefore, in order to obtain higher fermentation yields, many authors have studied the impact of the continuous removal of glucose and residual sucrose from the medium during the FOS conversion. Consequently, by increasing the fermentation yields a purer final product could be obtained (Nobre *et al.* 2012).

High FOS production yields adding glucose oxidase to the reaction media as been reported and many authors manage to control this effect and increase FOS production yield (up to 90 % ($g_{FOS}/g_{sucrose}$) – 98 % ($g_{FOS}/g_{sucrose}$)) by adding glucose oxidase to the reaction media which leads to the formation of gluconic acid, hence reducing glucose content in the media (Yun and Song 1993, Sheu *et al.* 2001, Lin and Lee 2008).

Other systems aiming glucose removal from the media have also been studied. For example, Sheu *et al.* (2002) reported a complex biocatalyst system with a bioreactor equipped with a microfiltration (MF) module. The system used mycelia of *A. japonicus* CCRC 93007 or *A. pullulans* ATCC 9348 with FFase activity and *Gluconobacter oxydans* ATCC 23771 with glucose dehydrogenase activity. Calcium carbonate slurry was used to control pH to 5,5, and gluconic acid in the reaction mixture was precipitated as calcium gluconate. Sucrose solution with an optimum concentration of 30 % (w/v) was employed as feed for the complex cell system, and high-content FOS was discharged continuously from a MF module. The complex system

produced more than 80 % ($g_{FOS}/g_{sucrose}$) FOS with the remaining being 5 % - 7 % glucose and 8 % - 10 % sucrose on a dry weight basis, plus a small amount of calcium gluconate.

Nishizawa *et al.* (2001) have achieved higher FOS yields with a simultaneous removal of glucose, using a membrane reactor system with a nanofiltration membrane, through which glucose permeated but sucrose and FOS not. FOS percentage of the reaction product was increased to above 90 % ($g_{FOS}/g_{sucrose}$), which was much higher than that of the batch reaction product (55 % ($g_{FOS}/g_{sucrose}$) – 60 % ($g_{FOS}/g_{sucrose}$)). Nonetheless, although these systems can be more efficient in terms of FOS production yield, it is important to notice that they are also more expensive.

With the same goal, Crittenden and Playne (2002) studied a different approach. In their study, immobilized cells of the bacterium *Zymomonas mobilis* were used to remove glucose, fructose, and sucrose from food-grade oligosaccharide mixtures, which were completely fermented within 12 h. The fermentation end products were ethanol and carbon dioxide, being a minimal amount of sorbitol also formed. Similarly to *Z. mobilis*, *Saccharomyces cerevisiae* also showed the ability to ferment some common mono- and disaccharides (fructose, glucose, galactose and sucrose) from a mixture of sugars while oligosaccharides with four or more monosaccharide units were not found to be fermented (Yoon *et al.* 2003, Goulas *et al.* 2007, Hernández *et al.* 2009). The microbial treatment seems to be a good alternative for increasing the percentage of FOS in a mixture through removal of mono- and disaccharides, being this process adequate to be used during the enzymatic synthesis of FOS. However, the use of microbial treatments implies a further step of purification for the removal of biomass and metabolic products formed during the fermentation, in order to obtain a FOS product with few contaminants, thus increasing the production cost. Also, the use of yeast treatment was found to modify the oligosaccharides composition (Sanz *et al.* 2005, Nobre *et al.* 2012).

Zuccaro *et al.* (2008) reported that recombinant FTases also have interesting biocatalytic properties. The combination of sucrose analogues, as novel substrates (substrate engineering), and highly active recombinant β-fructofuranosidase from *A. niger* (genetic engineering) provided a new powerful tool for the efficient preparative synthesis of tailor-made saccharides, 1-kestose and 1-nystose type.

1.6.3. FOS functional properties

FOS have a number of interesting properties. First, FOS have a low sweetness intensity since they are only about 1/3 as sweet as sucrose, supplying small amounts of energy, approximately 0–3 kcal/g of sugar substitute. This property is quite useful in the various kinds of foods where the use of sucrose is restricted by its high sweetness. Second, FOS are calorie free

since they are scarcely hydrolyzed by the digestive enzymes and not utilized as an energy source in the body, thus they are safe for diabetics. FOS are known to prevent the colonization of human gut by pathogenic microorganisms because it encourages the growth of beneficial bacteria. As FOS cannot be digested by small intestinal enzymes, they are fermented in the large intestine to selectively stimulate the growth of probiotic-like bacteria that are part of the commensal gut microbiota and then produce SCFAs, mainly acetate, propionate, and butyrate (Yun 1996, Yu Wang *et al.* 2010).

As previously mentioned, these compounds have the capacity of promoting a good balance of intestinal microbiota and decrease gastrointestinal infections. The beneficial physiologic functions of FOS in humans are briefly summarized below.

Protection against colon cancer

Prebiotics have been postulated to be protective against the development of colon cancer that is the second most prevalent cancer in humans. It is known that several species of bacteria commonly found in the colon produce carcinogens and tumor promoters from the metabolism of food components. There have been several studies on the use of prebiotics in cancer prevention, mainly focusing on animal models but the “dietary fiber hypothesis” for protection against colorectal cancer was advanced by Burkitt (1969), based on epidemiological evidence supporting a relationship between diet and colon health. These findings showed lower rates of colorectal cancer in Africa compared to industrialized Western countries, where traditional diets consisted of high amounts of unrefined fiber and high intakes of refined carbohydrates, respectively. (Manning and Gibson 2004, Lim *et al.* 2005a)

The two main types of fermentation that are carried out in the gut are saccharolytic and proteolytic. Saccharolytic activity is more favorable than a proteolytic fermentation due to the type of metabolic end products that are formed. The proximal colon is essentially a site of saccharolytic fermentation, whereas the more distal sees a depletion of available carbohydrate and more protein metabolism (Gibson 2004).The main end products of saccharolytic fermentation are the SCFAs, acetate, propionate and butyrate. All contribute towards the host’s daily energy requirements. Acetate is metabolized in systemic areas like muscle, while propionate is transported to the liver and used to generate ATP. Butyrate is an important source of energy for the colonocytes and is thought to have anti-tumor properties. Many studies were made to prove this and according to Kim *et al.* (1982) butyrate is a protector agent against colon cancer by promoting cell differentiation. Bugaut and Bentéjac (1993) reported that butyrate is used by the epithelial cells of the colon mucosa as energy source, being in addition a growth factor and more recent preclinical studies have reported that butyrate might be chemopreventive in carcinogenesis

(Scheppach and Weiler 2004). In addition to butyrate, propionate can have anti-inflammatory effects on colon cancer cells (Munjal *et al.* 2009).

On the other hand, the end products of proteolytic fermentation include nitrogenous metabolites (such as phenolic compounds, amines and ammonia) some of which are carcinogens. Many common diseases of the human large bowel arise in the distal colon, particularly colonic cancer. Most current prebiotics are of relatively small degree of polymerization (DP), the exception being inulin. It is thought that the oligosaccharides must be hydrolyzed by cell-associated bacterial glycosidases prior to uptake of the resultant monosaccharides. It is, therefore, reasonable to assume that the longer the oligosaccharide the slower the fermentation—and hence the further the prebiotic effect will penetrate more effectively throughout the colon. For example, long-chain inulin may exert a prebiotic effect in more distal colonic regions compared with the lower-molecular-weight FOS, which may be more quickly fermented in the saccharolytic proximal bowel (Gibson 2004, Manning and Gibson 2004).

Immunomodulatory effect

The functional foods are reported to enhance the immunity of the consumers. Indeed, the dietary components and their fermentation metabolites are in close contact with the gut associated lymphoid tissue (GALT) which is the biggest tissue in the immune system comprising 60 % of all lymphocytes in the body (Delgado *et al.* 2011, Saad *et al.* 2013). It contains Peyer's patches, lamina propria and intraepithelial lymphocytes forming a unique immune network. (Iijima *et al.* 2001, MacDonald 2003, Mowat 2003).

The presence of food in the small intestine may be necessary for adequate function and development of GALT; therefore, dietary carbohydrates are of major importance since they are the principal substrates for bacterial growth. It has been estimated that about 10–60 g/day of dietary carbohydrate reaches the colon acting like growth factor to particular commensal bacteria, which inhibit the adherence and invasion of pathogens in the colonic epithelia by competing for the same glycoconjugates present on the surface of epithelial cells, altering the colonic pH, favoring the barrier function, improving the mucus production, producing SCFAs and inducing cytokine production (Figure 1.4) (Korzenik and Podolskyl 2006).

Many researchers recognized the effect of oral administration of prebiotics. In 1997, Pierre *et al.* reported the development of GALT associated with inulin and FOS intake. Hosono *et al.* (2003) referred that inulin and FOS were able to modulate various parameters of the immune system, like the secretion of IL-10 and interferon (IFN-g) by Peyer's patch and Nakamura *et al*. (2004) recognized the immunoregulation of intestinal IgA, increase of polymeric immunoglobulin receptor (pIgR) expression in both the small intestine and the colon in infant mice. Schley and Field (2002) have also reviewed some evidence of immune-enhancing effects of dietary fibers and prebiotic.

Indeed, consumption of prebiotic fibers induces a variation of the intestinal microbiota which may potentially mediate immune changes, via the short-chain fatty acids produced from the fiber fermentation, direct interaction of lactic acid bacteria or bacterial cell wall or cytoplasmic components with immune cells in the intestine and finally by modulation of mucin production. Also, it was identified for the first time gene markers for physiological effects of a particular FOS on a host animal, making it possible to clarify the mechanisms of how the immunomodulatory effects of FOS are expressed in the body.

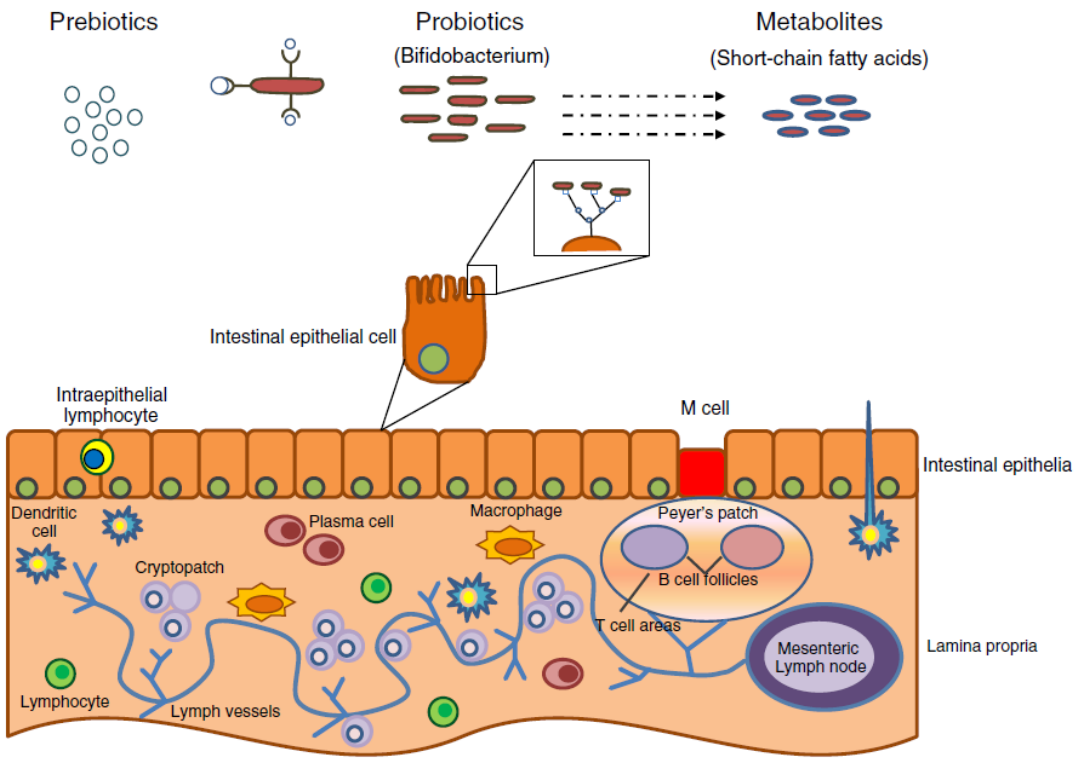


Figure 1.3. Interactions of prebiotics, probiotics and immune system in the intestinal mucosa. Prebiotics promote the growth of healthy microorganisms in intestinal environment, which display immunomodulatory functions, and inhibit the adherence and invasion of pathogens in the colonic epithelia (From Delgado *et al.* 2011).

Obesity and diabetes

Fiber intakes are associated with increased satiety and lower body weight. Beyond glycemic control, intestinal fermentation of fibers may also influence satiety. However, these mechanisms are not completely explained. One hypothesis is that the production of SCFAs, influence postprandial glucose response by reducing fat competition for glucose disposal. On the other hand, another hypothesis relies on the idea that fermentation SCFAs influences gut hormones

and gastric motility (Brighenti *et al.* 2006, Nilsson *et al.* 2008). Another scientific data also suggests that the decrease in food intake associated with prebiotics feeding in animals might be linked to the modulation of GI peptides involved in the regulation of food intake (Druce *et al.* 2004, Chaudhri *et al.* 2008).

Obesity and obesity related type II diabetes are typical diseases of the modern Western society. Current recommendations for the management of type II diabetes and obesity include an increase in dietary fiber intake. Dietary fiber's viscose and fibrous structure can control the release of glucose with time in the blood, thus helping in the proper control and management of diabetes mellitus and obesity (Bennett *et al.* 2006).

The health benefits of increased fiber consumption are well established; therefore, functional fibers like FOS, which can be easily added to traditionally low fiber foods, do warrant additional long-term study in the context of appetite control, as well as the promotion of health (Hess *et al.* 2011).

Improving mineral adsorption

Another observation of interest is the positive effect of the consumption of FOS on mineral absorption. Mineral deficiencies remain an important nutritional issue in the world. Calcium (Ca) intake in many under countries is below the recommended daily allowance, which leads to progressive bone loss. This particular effect of ingredients showing a prebiotic effect is indeed especially challenging because, among the bone builders, Ca is critical in achieving optimal peak bone mass and modulating the rate of bone loss associated with ageing, and is the most likely to be inadequate in terms of dietary intakes. Consequently, this specific property of prebiotics has been investigated extensively because if the mineral is inadequate during growth, the full genetic program for skeletal mass acquisition cannot be achieved. Then, if Ca intake is not enough to offset obligatory losses, acquired skeletal mass cannot be maintained, leading to osteoporosis, a major public health problem. Magnesium is the second most abundant intracellular cation in vertebrates, and its deficiency has been implicated as a risk factor for osteoporosis. Another mineral with a low intake is iron (Fe), which is a major cause of anemia. The importance of zinc (Zn) nurture for growth and development has been documented widely. Marginal Zn deficiency is suspected to be widespread in populations heavily dependent on cereal-based diets containing few animal products (Baba *et al.* 1996, Roberfroid *et al.* 2010, Yu Wang *et al.* 2010).

Several mechanisms have been proposed to elucidate the possible roles of FOS in improving mineral absorption. Even though mineral absorption generally occurs in the upper part of the intestine, it seems that, after consumption of FOS, the major site of action leading to improved bioavailability of minerals is the large intestine. The main action of FOS is mainly associated with their fermentation by resident microbiota. SCFAs decrease luminal pH and thus create an acidic

environment more favorable for mineral solubility (Gudiel-Urbano and Goñi 2002, Yu Wang 2010). Another way to contribute to the enhanced mineral absorption is the trophic effect of prebiotics on the gut (cell growth and functional enhancement of the absorptive area). It has been suggested that this is mediated by an increased production of butyrate and/or certain polyamines (Roberfroid 1993, Raschka and Daniel 2005).

Effects on serum lipid and cholesterol concentrations

There is interest in the food industry in developing functional foods to modulate blood lipids, such as cholesterol and triglycerides, because cardiovascular risk is the major public health concern in many countries and accounts for more deaths than any other disease or group of diseases (Manning and Gibson 2004, Qiang *et al.* 2009).

Many attempts have been made to control serum triacylglycerol (TAG) concentrations through the modification of dietary habits. The hypotriglyceridemic effect of non-digestible but fermentable carbohydrates', including resistant starch or FOS, has been described both in humans by Glore *et al.* (1994) and Jackson *et al.* (1999), and in animals by Tokunaga *et al.* (1986) and Yamamoto *et al.* (1999).

Ingredients showing a prebiotic effect are able to modulate hepatic lipid metabolism in rats or hamsters, resulting in changes in either TAG accumulation in the liver (steatosis) or serum lipids. The decrease in TAG synthesis and accumulation of dietary prebiotics compounds could be linked to several events. First, a decrease in glycemia could be part of the process, since glucose (together with insulin) is a driver of lipogenesis. Secondly, the SCFAs produced through the fermentation process could play a role in the regulation of lipid metabolism. The high proportion of propionate produced in the caecum, which reaches the liver through the portal vein, is, at least in animals, a key event in explaining a lower hepatic TAG synthesis (Delzenne and Kok 2001, Delzenne *et al.* 2002).

Among several studies, Levrat *et al.* (1994) and Fiordaliso *et al.* (1995) reported a decrease in total serum cholesterol after dietary supplementation with inulin (10 %) in mice or rats. This is accompanied by a significant decrease in the hepatic cholesterol content. The authors suggest that the decrease in serum cholesterol could reflect a decrease in TAG-rich lipoproteins.

1.6.4. FOS market data

In general, the total cost of developing a conventional new food product is estimated to be up to 1 or 2 million US$, while the development and marketing costs of functional food products may

exceed this level by far. Presently, the European market of functional food is dominated by gut health products. Germany, France, United Kingdom and the Netherlands account for around 2/3 of all sales of functional dairy products in Europe. Functional dairy products have shown an impressive growth bringing, for example, the market volume in Germany from around 5 million US$ in 1995 to 419 million US$ in 2000, of which 301 million US$ account for pro-, prebiotic and other functional yoghurts, and around 118 million US$ for functional drinks. The World demand for prebiotics is estimated to be around 167.000 tons and 390 million Euro. Among them FOS, inulin, isomalto-oligosaccharides, polydextrose, lactulose and resistant starch are considered the most popular. In 1995, the global market for FOS from sucrose was estimated to be 20.000 tons (Menrad 2003, Siró *et al.* 2008).

As previous mentioned, at an industrial scale FOS are produced enzymatically by two different processes (transfructosylation of sucrose or hydrolysis of inulin) which yield slightly different end products. The companies that commercially produce FOS from sucrose or inulin and their trade names are listed in Table 1.2. These FOS products are provided with a purity level above 95 % (Singh and Singh 2010, Nobre *et al.* 2012).

The enzymatic synthesis route to FOS using FTase from *A. niger* was first developed by Meiji Seika Kaisha Ltd., Japan to launch the commercial product Meioligo®. Then, this company also established a joint venture with Beghin-Meiji Industries, France to produce FOS marketed as Actilight®, and also with GTC Nutrition, USA to make FOS under the trade name NutraFlora® (Singh and Singh 2010). According to Meiji Holdings Co, Ltd Annual Report (2011), they presented a total net sales of almost 27 million US$ and operating incomes of 0,7 million US$.

Table 1.2. Commercially produced food-grade FOS.

Substrate	Manufacter	Trade name
Sucrose	Beghin-Meiji Industries, France	Actilight®
		Profeed®
	Cheil Foods and Chemicals Inc., Korea	Oligo-Sugar
	GTC Nutrition, USA	NutraFlora®
	Meiji Seika Kaisha Ltd., Japan	Meioligo®
	Victory Biology Engineering Co., Ltd., China	Beneshine™ P-type
Inulin	Beneo-Orafti, Belgium	Orafti®
	Cosucra Groupe Warcoing, Belgium	Fibrulose®
	Sensus, Netherlands	Frutalose®
	Nutriagaves de Mexico S.A. de C.V., Mexico	OLIFRUCTINE-SP®

Additionally, individual FOS molecules with purities varying between 80 % and 99 % are only available for analytical purposes. The main companies supplying 1-kestose (GF2), nystose (GF3), and 1^F-β- fructofuranosyl nystose (GF4) are Sigma Aldrich, Megazyme and Wako Chemicals GmbH (Nobre *et al.* 2012).

1.7. CONCLUSIONS

Modern consumers are increasingly interested in their personal health, and expect the foods they eat to be – beyond tasty and attractive – also safe and healthy. Non-digestible carbohydrates such as dietary fibers, oligosaccharides, and resistant starch have various physiologic functions and the effects of many non-digestible carbohydrates on wellbeing, better health and reduction of the risk of diseases have been well examined. Among functional oligosaccharides, FOS present important physicochemical and physiological properties beneficial to the health of consumers, and for this reason, their use as food ingredients has increased rapidly, presenting a significant growth on the functional food market all over the world. Europe nutraceutical market is expected to have a share over than 20 % until 2017. In view of the great demand for FOS as food ingredients, scope exists for screening and identification of novel strains capable of producing enzymes with transfructosylation activity and for developing improved less-expensive production methods. In this review it was demonstrated the beneficial effects of FOS and how they can play a key-role in the food market, bearing in mind that more effective less-cost production methods can be a main advantage in the food industry.

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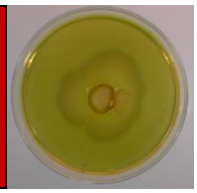
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CHAPTER 2:
SCREENING OF FUNGI WITH TRANSFRUCTOSYLATION ACTIVITY



"Make everything as simple as possible, but not simpler"

Albert Einstein.

Several microorganisms are reported to have transfructosylation activity due to FTase and/or FFase activities. However, the search of other fungi with transfructosylation activity remains a challenge. Therefore, a presumptive and indirect colorimetric plate assay for the evaluation of transfructosylation activity in fungi was developed, which involved the simultaneous determination in the same plate of glucose and fructose released from sucrose. This method was validated in more than 40 fungal strains from Micoteca da Universidade do Minho (MUM) collection, being identified transfructosylation activity in fungus that have never been reported as FOS producing microorganisms .

2.1. INTRODUCTION

2.2. MATERIALS AND METHODS

2.2.1. Reagents and enzymes

2.2.2. Microorganism and culture conditions

2.2.3. Screening procedure

2.3. RESULTS

2.4. DISCUSSION

2.5. CONCLUSION

2.6. REFERENCES

2.1. INTRODUCTION

FOS are widely used as a prebiotic, as they improve the health of the host due to their excellent biological and functional properties. They can be found in trace amounts in certain foods. However, mass production is limited by seasonal restrictions and the inherent inefficiencies of these natural systems (L'Hocine *et al.* 2000, Yoshikawa *et al.* 2006).

There is still a dispute in the scientific community regarding the nomenclature of FOS-producing enzymes. Some researchers use the term β-fructofuranosidase (FFase, EC. 3.2.1.26) (Nguyen *et al.* 1999, Sheu *et al.* 2001) whereas others designate it as fructosyltransferase (FTase, EC.2.4.1.9) (Yun *et al.* 1997, Sangeetha *et al.* 2004, Vandáková *et al.* 2004). The latter denomination is probably due to the fact that FTase activity was originally found from the side action in the course of FFase preparation when acting on high concentration of sucrose (Straathof *et al.*1986, L'Hocine *et al.* 2000). Transfructosylating activity, act on sucrose by cleaving the β (1, 2) linkage and transferring the fructosyl group to an acceptor molecule such as sucrose, releasing glucose. This reaction yields FOS, i.e. fructose oligomers mainly composed of 1-kestose (GF2), nystose (GF3) and 1^F-β- fructofuranosyl nystose (GF4) in which fructosyl units are bound at the β (2, 1) position of sucrose (Belghith *et al.* 2012).

According to BRENDA - The Comprehensive Enzyme Information System, FFase is a hydrolytic enzyme with some transfructosylation activity as well, while FTase only have transfructosylation activity. In the sucrose hydrolysis mechanism, a sucrose molecule is catalyzed in its monomers, glucose and fructose (Figure 2.1)

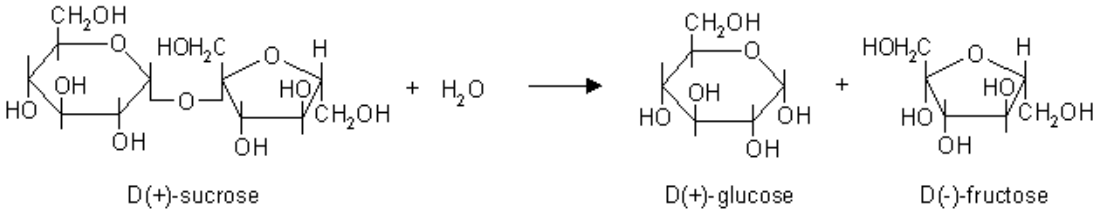


Figure 2.1. Sucrose hydrolysis catalyzed by FFase.

On the other hand, the enzymes with transfructosylation activity are capable to produce FOS according to the following mechanism (Figure 2.2.):

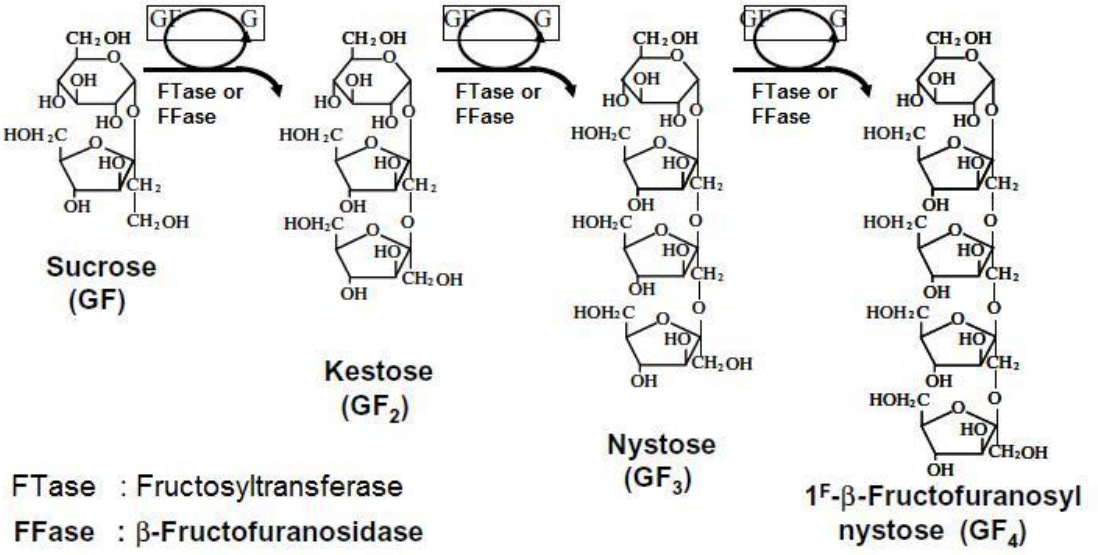


Figure 2.2. FOS production from sucrose by transfructosylation reaction catalyzed by FTase or FFase (adapted from Tokunaga 2004).

Glucose and fructose are reaction products associated with the synthesis of FOS and usually the transfructosylation activity of FFase and FTase is estimated by separate assay of glucose and fructose and determination of the excess of glucose produced over fructose (Cairns 1987).

The detection of glucose is based on the well-known glucose oxidase (GOD) – peroxidase (POD) system (Petzelbauer *et al.*1999, Barreto *et al.* 2000). The principle of this test is:

$$Glu + O_2 + H_2O \xleftrightarrow{GOD} Gluconic\ acid + H_2O_2$$

$$H_2O_2 + 4\text{-}aminoantipyrine + phenol \xrightarrow{POD} chromogen\ (pink)$$

For the determination of fructose, fructose dehydrogenase (FDH) is employed, as shown in the following reaction (Holmes 1997):

$$Fruc + MTT + Phenazine\ methosulfate \xrightarrow{FDH} 5\text{-}Keto\text{-}D\text{-}fruc + chromogen\ (blue)$$

The aim of the present study was to screen a serie of fungi from the collection of Micoteca da Universidade do Minho (MUM) and assess their capability to potentially produce FOS. In that sense, a presumptive and indirect colorimetric plate assay for screening relative transfructosylation activity of fungi by the simultaneous determination of glucose and fructose in the same sample was developed.

2.2. MATERIALS AND METHODS

2.2.1. Reagents and enzymes

Citric phosphate buffer (CPB) at pH 5.0 was used to prepare all stock solutions.

Stock solutions of MTT (Methylthiazolyldiphenyl-tetrazolium bromide) and 4-aminoantipyrine were prepared at 10 mg/mL and stored at 4 ºC. A solution of phenol at 20 mg/mL was prepared and stored at 4 ºC. Phenazine methosulfate was prepared at a concentration of 0.1 g/mL just prior to use.

FDH (Fructose dehydrogenase) and POD (horseradish peroxidase) were also prepared at concentrations of 250 U/mL and 125 U/mL, respectively. These stocks were stored at -20 ºC. GOD (glucose oxidase) was stored at 4 ºC, at a concentration of 1060 U/mL.

Under these conditions, both reagents and enzymes were stable.

2.2.2. Microorganism and culture conditions

Aureobasidium pullulans CCY 27-1-94 (Culture Collection of Yeasts, Bratislava, Slovakia) was used as standard microorganism, since it has transfructosylation activity (Vandáková *et al.* 2004). The fungi from MUM presented on table 2.1. were used as test microorganisms. The fungi were grown in Czapek Dox Media (CD) plates at 25 ºC for 5-7 days.

2.2.3. Screening procedure

One disc of mycelium (8 mm diameter) was cut from the edge of the colony of *A. pullullans* and placed onto CD agar plates and incubated at 25 ºC for 72 h.

After incubation, the colony was overlaid with soft agar (0.7 % (w/v)) at 40 ºC containing the biochemical reactive system, composed of: MTT 0,2 mg/mL, phenazine methosulfate 2,5 mg/L, 4-aminoantipyrine 0,16 mg/mL, phenol 1 mg/mL, FDH 2 U/mL, GOD 9 U/mL and POD 1 U/mL in CPB. After 10 min, the results were analyzed considering that the formation of pink (glucose) and blue (fructose) halos around the disc indicates the presence of the enzyme(s).

This same procedure was used for all the fungi listed on table 2.1.

Table 2.1. Fungi from MUM collection tested for relative transfructosylation activity.

Fungi	MUM number	Fungi	MUM number
Aureobasidium pullulans	94.10	*Penicillium aurantiogriseum*	02.11
Aureobasidium pullulans	97.04	*Penicillium aurantiogriseum*	02.40
Aspergillus aculeatus	03.11	*Penicillium chrysogenum*	03.18
Aspergillus flavus	00.06	*Penicillium commune*	02.20
Aspergillus flavus	00.29	*Penicillium commune*	02.22
Aspergillus ibericus	03.49	*Penicillium corylophilum*	02.38
Aspergillus ibericus	03.50	*Penicillium corylophilum*	03.35
Aspergillus ibericus	03.51	*Penicillium crustosum*	02.15
Aspergillus ibericus	04.68	*Penicillium crustosum*	02.30
Aspergillus ibericus	04.86	*Penicillium expansum*	02.03
Aspergillus japonicus	98.03	*Penicillium expansum*	02.14
Aspergillus niger	00.19	*Penicillium expansum*	99.26
Aspergillus niger	02.48	*Penicillium fellutanum*	02.35
Aspergillus niger	05.14	*Penicillium funiculosum*	02.32
Aspergillus niger	05.15	*Penicillium griseofulvum*	02.41
Aspergillus niger	05.16	*Penicillium griseofulvum*	02.46
Aspergillus niger	92.13	*Penicillium implicatum*	03.33
Aspergillus ochraceus	02.44	*Penicillium pinophilum*	02.47
Aspergillus ochraceus	93.02	*Penicillium roqueforti*	03.15
Aspergillus oryzae	98.04	*Penicillium spinulosum*	02.31
Aspergillus versicolor	02.33	*Penicillium spinulosum*	02.34
Aspergillus versicolor	02.37	*Penicillium variabile*	02.29
Aspergillus versicolor	03.28	*Penicillium thomii*	02.23

2.3. RESULTS

Three CD agar plates with *A. pullulans* were overlaid with glucose and fructose dye systems, separately and combined, to demonstrate the advantages of coupling the two dye systems (Figure 2.3).

The combined plate test was applied to the fungi from MUM (Table 2.1). The results analysis was made considering that *A. pullulans* is the model microorganism and exhibits a clearly positive answer, meaning, a reaction all over the plate (Figure 2.1. (f)).

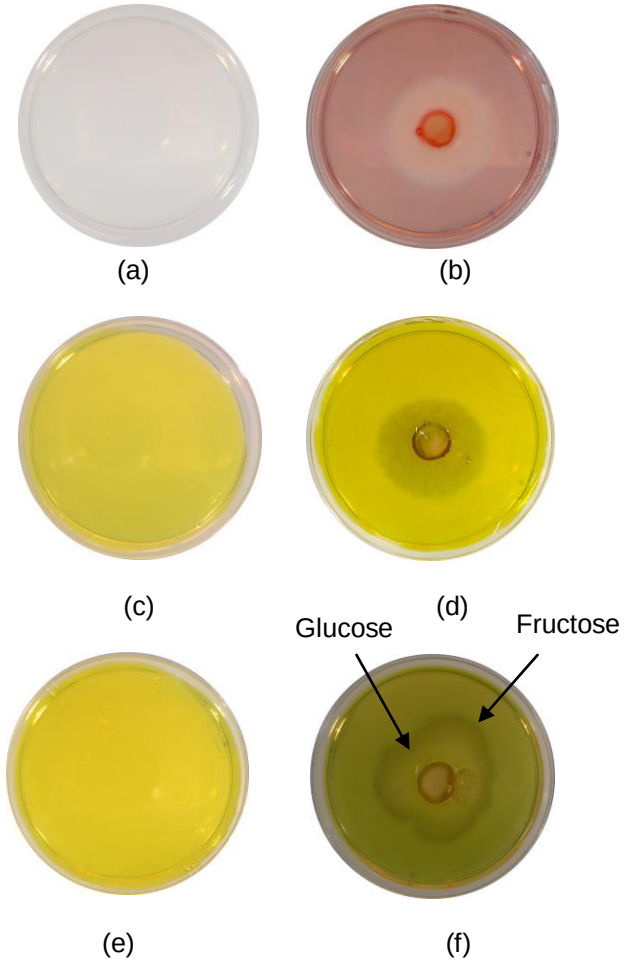


Figure 2.3. Detection of glucose (b), fructose (d) and glucose and fructose (f) in *A. pullullans* (standard microorganism) colonies grown in CD plates. Plates (a), (c) and (e) are the negative controls for each dye system.

Bearing in mind the visible differences between the coloration of the plates, indicating the different levels of transfructosylation activity of the different strains, the results were semi-quantified using an empirical numerical scale according to table 2.2 and figure 2.4.:

- 0: dye system does not react
- 1: dye system reacts in the border of the colony
- 2: dye system reacts beyond the border of the colony
- 3: dye system reacts all over the plate



3 (a) 2 (b) 1 (c)

Figure 2.4. Screening results of relative transfructosylation activity in *A. pullulans* (standard microorganism) (a), *A. ibericus* – MUM 03.49 (b) and *A. niger* – MUM 05.14 (b).

Table 2.2. Screening results of relative transfructosylation activity in Fungi from MUM collection tested.

Fungi	MUM number	Result	Fungi	MUM number	Result
Aureobasidium pullulans	94.10	3	*Penicillium thomii*	02.23	3
Aureobasidium pullulans	97.04	3	*Aspergillus ibericus*	03.49	2
Aspergillus aculeatus	03.11	3	*Aspergillus ibericus*	03.50	2
Aspergillus flavus	00.06	3	*Aspergillus ibericus*	03.51	2
Aspergillus flavus	00.29	3	*Aspergillus ibericus*	04.68	2
Aspergillus japonicus	98.03	3	*Aspergillus ibericus*	04.86	2
Aspergillus niger	00.19	3	*Aspergillus versicolor*	03.28	2
Aspergillus niger	02.48	3	*Penicillium aurantiogriseum*	02.40	2
Aspergillus niger	92.13	3	*Penicillium commune*	02.22	2
Aspergillus ochraceus	93.02	3	*Penicillium funiculosum*	02.32	2
Aspergillus oryzae	98.04	3	*Penicillium griseofulvum*	02.46	2
Penicillium aurantiogriseum	02.11	3	*Penicillium spinulosum*	02.31	2
Penicillium chrysogenum	03.18	3	*Aspergillus niger*	05.14	1
Penicillium commune	02.20	3	*Aspergillus niger*	05.15	1
Penicillium corylophilum	02.38	3	*Aspergillus niger*	05.16	1
Penicillium corylophilum	03.35	3	*Aspergillus ochraceus*	02.44	1
Penicillium crustosum	02.15	3	*Aspergillus versicolor*	02.33	1
Penicillium crustosum	02.30	3	*Aspergillus versicolor*	02.37	1
Penicillium expansum	02.03	3	*Penicillium fellutanum*	02.35	1
Penicillium expansum	02.14	3	*Penicillium griseofulvum*	02.41	1
Penicillium expansum	99.26	3	*Penicillium implicatum*	03.33	1
Penicillium roqueforti	03.15	3	*Penicillium pinophilum*	02.47	1
Penicillium spinulosum	02.34	3	*Penicillium variabile*	02.29	1

2.4. DISCUSSION

Several microorganisms have been reported to have transfructosylation activity due to FTase and/or FFase activities (e.g. *A. pullulans*, *A. niger*, *A. oryzae*, *A. japonicus*). However, the search for other fungi with higher transfructosylation activity remains a challenge (Madlová *et al.* 1999, Sangeetha *et al.* 2005a, Wang and Zhou 2006, Yoshikawa *et al.* 2008). Therefore, in order to identify new fungi with the potential to produce enzymes with transfructosylation activity, a presumptive and indirect colorimetric plate was developed.

In Figure 2.3, it can be seen that the dye system for detection of glucose reacts all over the plate, while the system to detect fructose only reacts around the colony. For this reason, the background of the plate with the coupling of the two dye systems becomes darker, as result of the overlapping of colors. However, in the last plate of this figure (f), the pink and blue halos around the colony are still visible, indicating the presence of enzymes with transfructosylation activity.

The screening method, when applied to fungi with enzymes with different transfructosylation activity, can show different results, as shown in Figure 2.4. These differences between the coloration of the plates, indicating the different levels of transfructosylation activity, allowed the semi-quantification of the enzymatic activity using an empirical numerical scale that goes from 0 (no reaction) to 3 (reaction all over the plate). After developing, implementing and validating this method, the relative transfructosylation activity of the fungi selected from MUM collection was evaluated (Table 2.2). The strains of *Aureobasidium*, *Aspergillus* and *Penicillium* were selected since they are frequently reported as FOS-producing enzymes microorganisms (Yun 1996, Sangeetha *et al.* 2005b).

The fungus *Aspergillus ibericus* was strategically tested since it was a new strain isolated from grapes, an environment with various types of sugars. *Aspergillus* species within section *Nigri* are important in biotechnological processes as well as biodeterioration. Species such as *A. niger* have a GRAS status from the FDA due to its extensive commercial and industrial uses. *Aspergillus* species in the section *Nigri* have proved to be particularly significant in the production of ochratoxin A (OTA). Nevertheless, it's important to notice that *A. ibericus* strains do not produce OTA, conferring this strain special interest for biotechnological exploration, since many metabolites with commercial value are produced by other species in the *Nigri* section (Serra *et al.* 2006). Therefore, even though it did not demonstrate the maximum relative transfructosylation activity, the *A. ibericus* strains tested are microorganisms with potential to produce FOS, since they are new strains with transfructosylation activity that have never been reported as FOS-producing microorganisms.

Analyzing Table 2.2, all *Aspergillus* strains from MUM collection with maximum relative transfructosylation activity, have already been reported as FOS producing microorganisms. Nemukula *et al.* (2009) described the synthesis of FOS by FTase from *A. aculeatus* and

Sangeetha *et al.* (2003) have reported transfructosylation activity in *A. flavus*. *A. ochraceus* have also been identified as a FOS-producing microorganism by Guimarães *et al.* (2007). On the other hand, *A. niger* (Wallis *et al*. 1997, L'Hocine *et al.* 2000), *A. japonicus* (Chen and Liu 1996, Mussato *et al.* 2009) and *A. oryzae* (Sangeetha *et al.* 2004, Kurakake *et al.* 2008) FFase and/or FTase activities have been extensively studied. Nevertheless, it's important to notice that even though *A. niger* is widely reported as a FOS-producing microorganism, not all *A. niger* strains tested presented high levels of transfructosylation activity.

Even though *Penicillium* sp. are reported as fungi capable of producing enzymes with transfructosylation activity, there are only few studies of FOS production with it. Yun (1996) have studied FOS production by *P. spinulosum* but the other *Penicillium* strains tested and identified as having maximum relative transfructosylation activity have not been reported as FOS-producing microorganisms. *P. aurantiogriseum*, *P. chrysogenum*, *P. commune*, *P. corylophilum*, *P. crustosum*, *P. expansum* and *P. roqueforti* showed great potential as fungi with high transfructosylation activity for FOS production. Recently, Prata *et al.* (2010) and Mussato *et al.* (2012) have reported FOS production by *P. expansum* (MUM 02.14) identified in this study and have obtained high FOS production yields.

2.5. CONCLUSION

Screening for new strains is hard work due to the large number of evaluations. The coupling of the two dye systems proved to be a method suitable for screening a large number of fungi due to its simplicity, reproducibility and rapidity. This method also gives a relative quantitative idea of the transfructosylation activity of different fungi species, becoming a new advantageous tool for identifying potential novel sources of FTase and/or FFase.

Fungal strains from the *Aspergillus* and *Penicillium* species, which has not been reported so far for FTase and/or FFase production, have been identified as fungus with relative transfructosylation activity, showing potential as FOS-production microorganisms.

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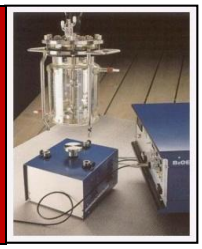
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CHAPTER 3:
OPTIMIZATION OF FOS PRODUCTION BY AUREOBASIDIUM PULLULANS USING RESPONSE SURFACE METHODOLOGY



"When you make the finding yourself - even if you're the last person on Earth to see the light - you'll never forget it."

Carl Sagan.

This chapter describes the optimization of fermentation conditions (temperature and agitation speed), in an integrated one-stage method, for maximization of FOS production via sucrose fermentation by *Aureobasidium pullulans* using experimental factorial design and response surface analysis. The optimization procedure allowed a significant improvement of the total FOS production yield using a one-stage process, being obtained a production yield of 64,1 ± 0,0 %($g_{FOS}/g_{sucrose}$) and process productivity of 2,67 ± 0,05 g_{FOS}/L.h, within 48 h of fermentation, under the fermentation conditions of T = 32 ºC and agitation speed = 385 rpm.

3.1. INTRODUCTION

3.2. MATERIALS AND METHODS

3.2.1. Microorganism and culture conditions

3.2.2. Experimental design and data analysis

3.2.3. Sugar analysis

3.2.4. Cell growth

3.3. RESULTS

3.3.1. Effects of the fermentation conditions on FOS production

3.4. DISCUSSION

3.5. CONCLUSION

3.6. REFERENCES

3.1. INTRODUCTION

Currently, it is widely recognized that the commensal bacterial community of human intestinal tract has a great impact on the gastrointestinal function, and consequently on human health and well-being. Under this scope, in the recent years consumers have been increasingly confronted with the so-called functional food products among which probiotics and prebiotics, such as FOS, constitute the most popular examples (Gibson *et al.* 2004, De Preter *et al.* 2011).

FOS can be produced by the action of enzymes with transfructosylation activity (i.e. FTAse (EC 2.4.1.9) and/or FFase (EC 3.2.1.26)) derived from plants and microorganisms. Most of these enzymes have been found in fungi such as *Aureobasidium* spp., *Aspergillus* spp. and *Penicillium* spp. (Sangeetha *et al.* 2004a, Dominguez *et al*. 2006, Mussatto *et al*. 2009a).

Conventionally, FOS production is a two-stage process, in which the first stage consists in the microbial production of the enzyme with transfructosylation activity, and the second stage consists in the reaction of the extracted enzyme with sucrose (substrate) to produce FOS under controlled conditions (Mussatto *et al.* 2009b, Sangeetha *et al.* 2005a). FOS production by fungi using bioreactors in a one-stage process is a great opportunity, as using whole cells as biocatalysts, either immobilized or free, eliminates the step of FOS-producing enzyme purification from the cell extracts. Nevertheless, maximization of FOS yields can be affected by several factors that are involved in the whole process (one-stage process) (Chien *et al*. 2001, Mussatto *et al.* 2009a, Sangeetha *et al.* 2005b).

The classical method of optimization involves changing one variable at a time, keeping the others at fixed levels. Being single dimensional, this laborious and time consuming method often does not guarantee determination of optimal conditions, neither takes into account possible interactions among various operational factors. Experimental design and optimization are tools that enable building models and evaluating the significance of the different factors considered, as well as their interactions. Furthermore, with these models, a small number of experimental trials are used to search the optimal factor levels that conduct to the desired response (Lundstedt *et al.* 1998, Montgomery 1997).

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. Is also has important applications in the design, development, and formulation of new products, as well as in the improvement of existing products design. The most extensive applications of RSM are in the industrial world, particularly in situations where several input variables potentially influence some performance measure or quality characteristic of the product or process. This performance measure or quality characteristic is called response. In addition, it is a good way to graphically illustrate the relation between different experimental variables and the responses. In the first screening it is

recommended to evaluate the result and estimate the main effects of the variables according to a linear model. After this evaluation, the variables that have the largest influence on the result are selected for new studies. Thus, a large number of experimental variables can be investigated without having to increase the number of experiments to the extreme (Montgomery 1997, Myers *et al.* 2009).

Therefore, the aim of this work is to optimize the operation parameters, namely temperature and agitation speed, that maximize the total FOS production, using a 2^k full-factorial central composite experimental design. According to the factorial design, 22 experiments plus 3 central points were performed. Additionally, 4 other points were also included in the set of experiments to make the central composite design rotatable, setting the distance from the central design equal to the square root of 2.

3.2. MATERIALS AND METHODS

3.2.1. Microorganism and culture conditions

The fungus *Aureobasidium pullulans* CCY 27-1-94 (Culture Collection of Yeasts, Bratislava, Slovakia), being a fungus like yeast, cannot be grown in liquid medium from spores. Therefore, the fungus was stored at -20 ºC in liquid medium with glycerol (17 % (v/v) (pre-inoculum)) after the following procedure: five discs of mycelium (8 mm diameter) were cut from the edge of the colony of *A. pullulans* kept in Czapek Dox Media (Oxoid, UK) plates and grown for 5 days at 28 ºC and 140 rpm in inoculation medium containing sucrose (100 $g.L^{-1}$), $NaNO_3$ (10 $g.L^{-1}$), $FeSO_4.7H_2O$ (0,01 $g.L^{-1}$), K_2SO_4 (0,35 $g.L^{-1}$), KCl (0,5 $g.L^{-1}$), KH_2PO_4 (5 $g.L^{-1}$) and $MgSO_4.7H_2O$ (0,5 $g.L^{-1}$), with an initial pH of 6.5. The inoculum was prepared by transferring 10 mL of pre-inoculum into the inoculation medium and subsequently it was grown for 3 days at 28 ºC and 140 rpm. The inoculum (3 % (v/v)) was transferred to the fermentation medium containing sucrose (200 $g.L^{-1}$), $NaNO_3$ (20 $g.L^{-1}$), $FeSO_4.7H_2O$ (0,01 $g.L^{-1}$), K_2SO_4 (0,35 $g.L^{-1}$), KCl (0,5 $g.L^{-1}$), KH_2PO_4 (5 $g.L^{-1}$) and $MgSO_4.7H_2O$ (0,5 $g.L^{-1}$). In the experimental design assays, optimization of the fermentation conditions was performed by varying the temperature and the agitation speed.

3.2.2. Experimental design and data analysis

The optimal temperature and agitation speed levels for maximizing the total FOS production were studied using a 2^k full-factorial design with 2 factors and 3 central points, according to Table 3.1.

Based on preliminary experiments (data not shown) and studies reported by other authors, pH was found to have no significant effect on total FOS production yield so it was not considered as an optimization parameter (Madlová *et al.* 1999).

Table 3.1. Experimental range and levels of the factors tested in the 2^2 full factorial central composite design.

Factor	Range and levels of factors				
	$-2^{1/2}$	-1	0	+1	$+2^{1/2}$
X_1 - Temperature (ºC)	21	25	35	45	49
X_2 – Agitation speed (rpm)	110	150	250	350	390

An additional central composite design was used to optimize those experimental conditions by means of a response surface methodology. Therefore, 4 extra experiments were carried out to rotate the central composite design, setting the distance from the central design equal to the square root of 2. The parameters ranges were chosen according to previous data on the optimum conditions for biomass growth, enzyme production and activity for this microorganism (Yun *et al.* 1997, Shin *et al.* 2004a, Vandáková *et al.* 2004). The two independent factors (temperature and agitation speed) were studied at 5 levels ($-2^{1/2}$, -1, 0, +1 and $+2^{1/2}$) and a set of 11 experiments were carried out randomly according to the combinations presented in Table 3.2.

Table 3.2. Experimental design of the 2^2 full factorial central composite design used for the optimization of the total FOS production.

Run	x_1	x_2
1	0	0
2	0	0
3	+1	-1
4	-1	+1
5	-1	-1
6	0	0
7	+1	+1
8	$-2^{1/2}$	0
9	$+2^{1/2}$	0
10	0	$-2^{1/2}$
11	0	$+2^{1/2}$

For the statistical treatment, the factors were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \quad i = 1,2 \qquad \text{(Equation 3.1)}$$

where x_i is the coded value of the independent factor, X_i is the real value of the independent factor, X_0 is the real value of the independent factor at the central point and ΔX_i is the step change value.

It is expected that the behaviour of the system could be explained by a second-order equation, used for predicting the optimal total FOS yield point (y), based on the coded values of the independent factors (x_i):

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon \qquad \text{(Equation 3.2)}$$

where y is the predicted response; the β's are parameters whose values are to be determined using multiple linear regression model (MRLM), being β_0 the intercept, β_1 and β_2 the linear coefficients, β_{11} and β_{22} the quadratic coefficients and β_{12} the interaction coefficient; x_1 and x_2 are the coded independent factors and ε is a random error term of the regression model, which should be independent and follow a normal distribution, in accordance to the requirements of the MRLM.

Design-Expert 6.0.6. (Trial version) and Statistical Package for Social Sciences (SPSS, version 14) were used for the experimental design and regression analysis of the experimental data. The significance of the regression model was evaluated using analysis of variance (ANOVA). The quality of the fit obtained using the regression model equation was statistically checked by the magnitude of two diagnostic residuals: the multiple or adjusted coefficient of determination (R^2 or R^2_{adj}, respectively) and the predicted coefficient of determination (Q^2). The R^2-values describe the goodness of fit, giving an idea of how well current runs can be reproduced by the mathematical model. The Q^2-value describes the goodness of prediction, showing how well new experiments can be predicted using the mathematical model. R^2 and Q^2 values higher than 0,75 and 0,60 indicate that the model is good, and Q^2 values lower than 0,25 indicate that the model is useless (Mandenius and Brundin 2008).

The discrimination ability of the model was also inferred by calculating the adequate precision value, which compares the range of the predicted values at the design points to the average prediction error. A value greater than 4 indicates an adequate model discrimination. The significance of the regression coefficients was tested using a t-test. Simultaneously, the independence and the normal distribution of the regression model error were studied using the Durbin-Watson and the Shapiro-Wilk's tests, respectively. For the former, a statistic value of 2,0 ± 0,2 will guarantee the independence of the error term. Also, the required non multi-collinearity condition between the independent variables was evaluated using the variance inflation factor (VIF) and the condition index statistics. The independent variables will be non-collinear if values

lower than 10 and 30, respectively, are obtained. Moreover, the presence of outliers was also checked by means of the outlier t-test, which tests if a run (experiment result) is consistent with the other runs, assuming that the model developed is suitable. Values higher than 3,5 indicate the presence of an outlier. Finally, the contour plots obtained from the second-order model proposed were also used to infer about the optimal experimental conditions keeping the independent factors within the experimental range studied. To validate the optimal operation condition levels that maximize total FOS production given by the model, 3 additional experiments were carried out.

3.2.3. Sugar analysis

Sugar concentrations in the fermentation broth were analyzed by high-performance liquid chromatography (HPLC). A modular liquid chromatograph (Jasco) equipped with a Prevail Carbohydrate ES column (5 µm, 250 x 4,6 mm) from Alltech was used, at room temperature. Elution was achieved using a mixture of acetonitrile and 0,04 % ammonium hydroxide in water (70:30 v/v) at a flow rate of 1,0 $mL.min^{-1}$. The response of the refractive index detector was recorded and integrated using the Star Chromatography Workstation software (Varian) (Dias *et al.* 2009).

3.2.4. Cell growth

Cellular growth was quantified by biomass determination (dry weight) using a microwave oven. Briefly, 10 mL of of the fermentation broth was filtered through a 0,45 µm filter and dried in a microwave oven for 15 min at 150 W. The filter was then placed in a desiccator to cool for 10-15 min and afterwards it was weighted. Also, all filters were dried and weighted before the filtration (Olsson and Nielsen 1997). This procedure was done in triplicate for each fermentation broth sample.

3.3. RESULTS

3.3.1. Effects of the fermentation conditions on FOS production

The factorial design enabled the identification of the fermentation conditions that play a significant role on the total FOS production. The factorial design, experimental data and predicted results are given in Table 3.3.

Table 3.3. Experimental design and results of the 2^2 full factorial central composite design used for the optimization of the total FOS production.

Run	x_1	x_2	% FOS (w/w)	
			Observed	Predicted
1	0	0	61,9	61,4
2	0	0	63,7	61,4
3	+1	-1	52,9	50,0
4	-1	+1	60,9	61,3
5	-1	-1	56,1	56,4
6	0	0	58,7	61,4
7	+1	+1	58,1	54,9
8	$-2^{1/2}$	0	55,9	54,9
9	$+2^{1/2}$	0	42,1	45,8
10	0	$-2^{1/2}$	56,3	57,5
11	0	$+2^{1/2}$	62,9	64,4

MLRM was performed to fit the response function (y corresponds to the % total FOS in w/w) with the experimental data, allowing to obtain a significant second-order polynomial model. This model was established taken into account that the global model, as well as their coefficients must be statistically significant at the usual significance levels (5 % or 10 %), however keeping in mind that (Montgomery 1997, Mandenius and Brundin, 2008):

(i) Effects are disregarded from the analysis if the hierarchy of the model is not affected.

(ii) Effects are not removed if the final model is a ridge system where several local optimum points exist and the real stationary point is not inside the region of exploration for fitting the second-order model.

(iii) The ensure that the final model has a satisfactory prediction performance the predicted coefficient of determination value must be greater than 0,25.

Bearing in mind the above-mentioned considerations, a MLRM was established to fit the response function based only on the linear and quadratic parameters, allowing deriving a reduced second-

order model. The statistical significance of the model and the coefficients of the response surface were evaluated using the F-test analysis of variance (ANOVA) and student t-test, respectively (Tables 3.4 and 3.5):

$$y = 61{,}433 - 3{,}190x_1 + 2{,}417x_2 - 5{,}543x_1^2 - 0{,}241x_2^2 \quad \text{(Equation 3.3)}$$

Table 3.4. Analysis of variance (ANOVA) for the second-order model determined for % total FOS (w/w).

Source	SS	DF	MS	*F*-value	*P*-Value
Model	313,44	4	78,36	9,34	0,0095
Residual	50,30	6	8,39		
Lack of fit	37,51	3	9,38	1,46	0,4447
Pure error	12,83	2	6,41		
Total	363,78	10			

Table 3.5. Regression analysis using the 2^2 full factorial central composite design.

Factor	β's coefficients	Standard errors	*t*-values	*P*-values
Intercept	61,433	±1,673	36,727	<0,001*
x_1	-3,190	±1,024	-3,114	0,021*
x_2	2,417	±1,024	2,359	0,056**
x_1^2	-5,543	±1,219	-4,545	0,004*
x_2^2	-0,241	±1,219	-0,198	0,850

(*) Statistically significant at a 5% significance level
(**) Statistically significant at a 10% significance level

The model was found to be statistically significant (P=0,0095) and did not show lack-of-fit (P=0,4447). Moreover, it showed an acceptable determination coefficient (R^2=0,862) that explains 86,2 % of the response variability, as well as a satisfactory adjusted determination coefficient ($R^2_{adjusted}$=0,769). Also, a satisfactory prediction determination coefficient was obtained (Q^2=0,442) showing that the prediction abilities of the proposed model are acceptable. Furthermore, a small variation coefficient was obtained (equal to 5,54 %) and the model showed an adequate precision (equal to 9,49), which means that the proposed model can be used to navigate the design space and for optimization purposes. Finally, as required by the MRLM, the random error term (ε) of the regression model was found to be independent (d_{DW}=1,868) and to follow a normal distribution

(P=0,861, for the Shapiro-Wilk's test). In addition, no outliers were identified in the experimental data set, being, in general, the values of the outlier-t test lower than 3,5. Also, no statistical evidences of multi-collinearity were found since the VIF and condition indexes calculated for all the terms included in the model (linear and quadratic terms) varied from 1,000 to 1,095 and were lower than 3,6, respectively.

The three-dimensional response surface described by the model (Equation 3.3), for the region experimentally studied, is represented in Figure 3.1. A comparison between experimental yield data and the model predicted values is shown in Figure 3.2. As can be seen, the yield values estimated by the model are in good agreement with the experimental ones.

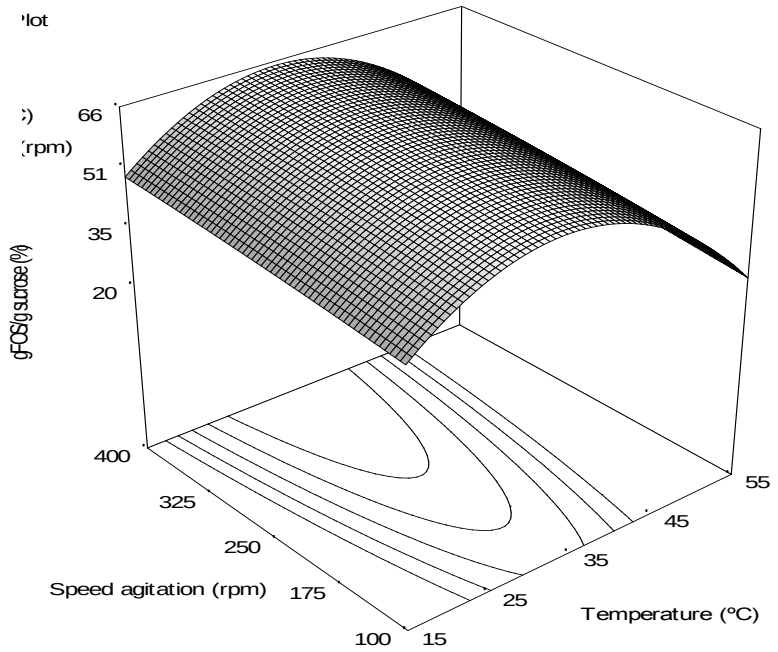


Figure 3.1. Response surface described by the model in the region explored experimentally.

Moreover, the fitted surface (Figure 3.1) shows a possible maximum point based on the contour plots analysis, at a temperature of 32 ºC and a speed of agitation of 385 rpm. Under these conditions, the corresponding maximum yield of total FOS predicted by the model was 64,7 ± 8,8 %($g_{FOS}/g_{sucrose}$) (95 % confidence).

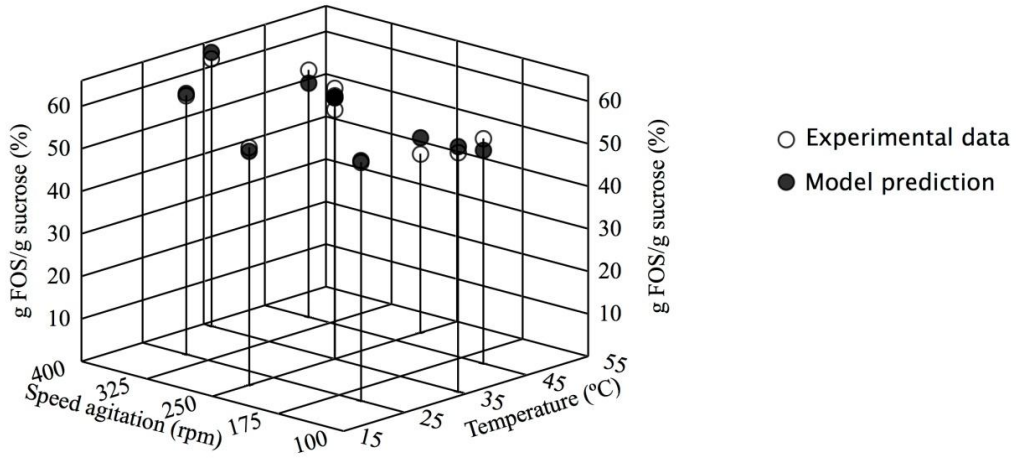


Figure 3.2. Comparison between experimental results and the values predicted by the model.

To validate these results, 3 independent fermentations were conducted at the optimized conditions (Figure 3.3). On these experiments, an average total FOS yield of 64,1 ± 0,0 % ($g_{FOS}/g_{sucrose}$) (1-kestose; 43,6 $g_{1\text{-}kestose}/g_{sucrose}$, nystose; 20,6 $g_{nystose}/g_{sucrose}$) was obtained, which is similar to the predicted value, resulting in a productivity of 2,67 ± 0,05 g_{FOS}/L.h. The HPLC chromatogram of the fermentation medium in the maximum yield production point is also presented in Figure 3.4 in order to demonstrate FOS (in this case, 1-kestose and nystose) and sucrose separation and quantification.

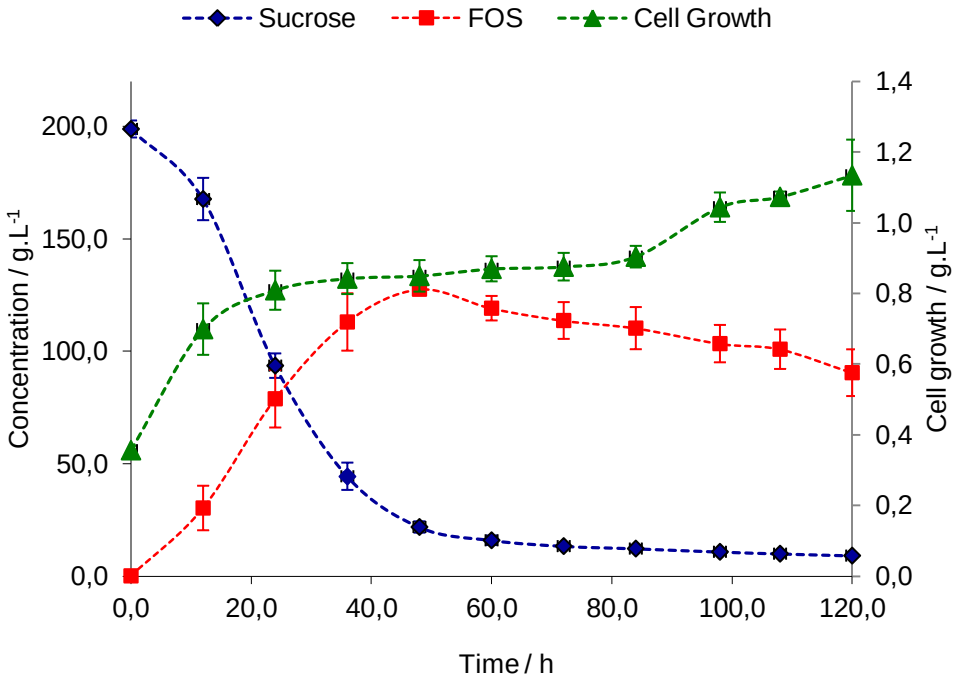


Figure 3.3. Kinetics of cell growth (cell growth/g.L^{-1}), total FOS production and sucrose consumption (concentration/g.L^{-1}) for *A. pullulans* using the optimized conditions.

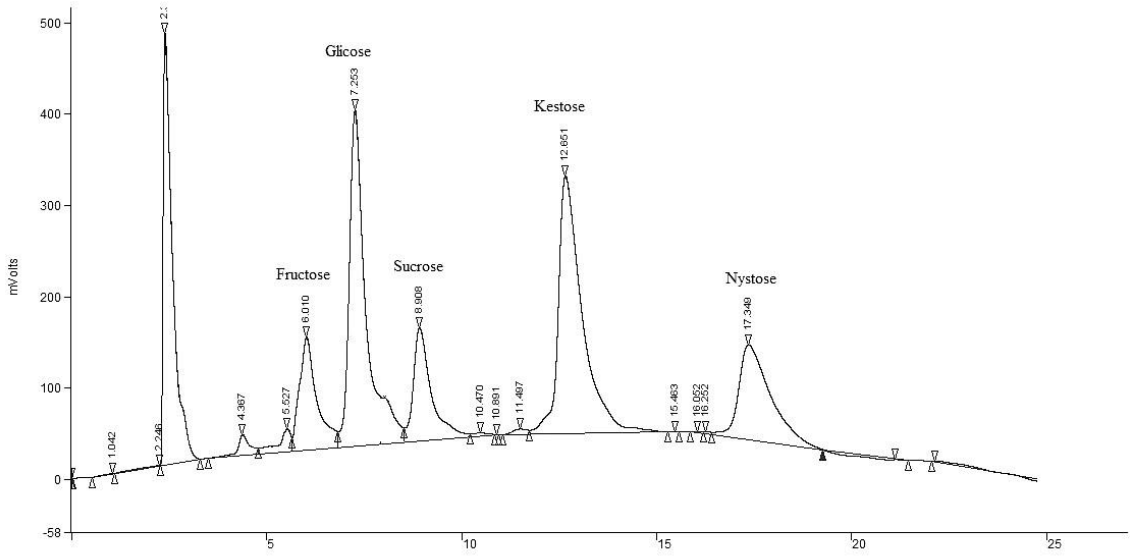


Figure 3.4. HPLC chromatogram of the fermentation medium in the FOS maximum yield production point (64,1 ± 0,0 $g_{FOS}/g_{sucrose}$) at the optimized fermentation conditions (temperature = 32 ºC, speed of agitation = 385 rpm).

3.4. DISCUSSION

The base concepts in nutrition science have been changing in the last years and there has been an increasing interest in the use of oligosaccharides in diets, such as FOS (Roberfroid 2007). *A. pullulans* is a FOS-producing microorganism, and many studies have reported high total FOS production yields using the enzyme FFase produced by this fungus (Sangeetha *et al.* 2004a, Shin *et al.* 2004a, Vandáková *et al.* 2004; Lateef *et al.* 2007, Yoshikawa *et al.* 2008). Usually, FOS production is a two-stage process, in which the first stage consists in the enzyme production by fermentation, and the second stage in the reaction of the enzyme with sucrose to produce FOS (Yun 1996, Yun *et al.* 1997, Sangeetha *et al.* 2004a, Sangeetha *et al.* 2005a).

The current study focused on the maximization of total FOS production in a one-stage process by *A. pullulans* using response surface methodology to optimize the operation parameters, namely temperature and agitation speed. Statistical tools have been commonly used to optimize fermentation parameters and consequently to maximize the production yields of a number of biotechnological compounds with commercial value as is the case of prebiotics (Vandáková *et al.* 2004, Prata *et al.* 2010, Torres *et al.* 2010, Aachary and Prapulla 2011).

The total FOS production yield obtained in the optimum operation conditions was found to be in accordance with the expected value predicted by the model, showing that the model was well fitted to the experimental data and thus describing accurately the studied region.

Table 3.6 presents a comparison between FOS production yield obtained in this experiment and other studies reported in the literature. Analyzing the data collected it is interesting to notice that the yields obtained in the current work are competitive when compared to the one's presented in other studies that are mostly two stage process using FTase and/or FFase produced by submerged fermentation (SmF). As regards *A. pullulans* as FOS-producing microorganism, the yield obtained in this study is higher than the others reported with the main advantage of being a one stage-process, hence eliminating enzyme purification costs and operation time. When compared with other enzyme sources, some fungi show even higher production yields. Nevertheless, it is important to notice that being *A. pullulans* a fungus like yeast, its growth and behavior in a SmF is friendlier when compared with filamentous fungi, which turn the fermentation medium more dense.

The *A. ibericus* strain was a microorganism identified in the screening procedure and, as it was a new *Aspergillus* strain recently isolated, its use as a FOS-producing microorganism is a novelty among the scientific community. In optimized fermentation conditions it also showed an interesting FOS production yield when compared with other *Aspergillus* strains.

Table 3.6. FOS yields obtained using FTase and/or FFase from various fungal strains.

Microorganism	Production Process	Reaction Time (fungus cultivation + enzymatic reaction)	Production Yield (% ($g_{FOS}/g_{sucrose}$))	Reference
Aureobasidium pullulans	Two stage process using isolated intracellular enzyme produced by SmF	96 h + 25 h	58,0	Yun and Song 1993
	Two stage process using extracellular enzyme produced by SmF	48 h + 24 h	55,9	Sangeetha *et al.* 2004a
	Two stage process using culture broth homogenate produced by SmF	96 h + 12 h	56,6	Sangeetha *et al.* 2004a
	Two stage process using isolated intracellular enzyme produced by SmF	72 h + 24 h	53,0	Shin *et al.* 2004a
	Two stage process with immobilized cell (SmF)	72 h + 24 h	44,0	Shin *et al.* 2004b
	Two stage process with isolated intracellular FTase produced by SmF	24 h + 9 h	59,0	Lateef *et al.* 2007
	Two stage process using crude FFase preparation produced by SmF	24 h + 24 h	62,0	Yoshikawa *et al.* 2008
	One stage process - present study - SmF	**48 h**	**64,1**	**Dominguez *et al.* 2012**
Aspergillus ibericus	One stage process - SmF	24 h	61,0 - 64,0	Gomes 2009*
Aspergillus japonicus	Two stage process using immobilized FFase produced by SmF	28 h + 17 h	61,0	Chiang *et al.* 1997
	Two stage process with isolated intracellular FFase produced by SmF	72 h + 24 h	55,8	Wang and Zhou 2006
	One stage process using corn cobs as the solid support - SSF	21 h	66,0	Mussato *et al.* 2009a*
	One stage process - SmF	24 h	61,0	Mussato *et al.* 2009a*
	One stage process using coffee silverskin as the solid support - SSF	16 h	70,0	Mussato and Teixeira 2010*
Aspergillus niger	Two stage process with isolated intracellular and extracellular FFase produced by SmF	24 h + 72 h	24,0 - 26,0	Nguyen *et al.* 1999
	Two stage process using washed cells produced by SmF	92 h + 8 h	70,0	Madlová *et al.* 1999
Aspergillus oryzae	Two stage process using extracellular enzyme produced by SmF	120 h + 12 h	54,1	Sangeetha *et al.* 2004a
	Two stage process using culture broth homogenate produced by SmF	96 h + 12 h	53,2	Sangeetha *et al.* 2004a
	Two stage process FTase produced by SSF, using corn germ supported as the solid support	120 h + 8 h	60,0	Sangeetha *et al.* 2004b
	Two stage process using extracellular enzyme produced by SmF	24 h + 18 h	57,4	Sangeetha *et al.* 2005a
	Two stage process using whole cells produced by SmF	24 h + 48 h	53,0	Sangeetha *et al.* 2005b
Penicillium expansum	One stage process - SmF	36 h	58,0	Prata *et al.* 2010*
	One stage process using synthetic fiber as the solid support - SFF - Repeated batch fermentation	60 h – three cycles	87,0-1st (36 h) 72,0-2nd (48 h) 44,0-3rd (60 h)	Mussato *et al.* 2012*

* Fungal strain identified in the screening procedure (Chapter 2).

There are many reports on the production of FTase and/or FFase by SmF using *Aspergillus* spp, *Penicillium* spp and *Aureobasidium* spp. However, few studies have been conducted on the production of these enzymes by solid state fermentation (SSF) (Sangeetha *et al.* 2004b). SSF is defined as a process in which microorganisms are grown on solid substrates in the absence of free water (Lagemaat and Pyle 2001). Agro-industrial residues are generally considered the best substrates for SSF processes, especially for enzyme production. Some of the substrates used include sugarcane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soy hull, corncobs, banana waste, tea waste, cassava waste, apple pomace, among others. The production of enzymes by SSF has potential advantages over SmF, with respect to simplicity in operation, high productivity fermentation, concentrated product formation and less favorable growth of contaminants (Balasubramaniem *et al.* 2001, Sangeetha *et al.* 2004b, Mussato *et al.* 2010). Mussato and Teixeira (2010) reported a 70 % ($g_{FOS}/g_{sucrose}$) production yield in 16 h, by SSF with an *Aspergillus japonicus* strain that has been identified as a FOS-producing enzyme during the screening procedure (Chapter 2), using coffee silverskin as the solid support. In this same study, when the fermentation medium was supplemented with micronutrients even higher production yields were obtained, reaching 100 % in 16 h, using corn cobs as solid support material.

By comparing total FOS production yield obtained in the current work, using a one-stage process and the one's obtained with the strains isolated in the screening procedure (Chapter 2) with values reported by other authors using a two-stage process, it is interesting to notice that higher production yields were herein obtained, with the main advantage of reducing operational costs and time. With the optimization procedure described, an improvement of the total FOS production yield using a one-stage process was obtained, achieving a production yield of 64,1 ±0,0 % ($g_{FOS}/g_{sucrose}$), in 48 h of fermentation, resulting in a productivity of 2,67 ± 0,05 g_{FOS}/L.h.

3.5. CONCLUSION

In summary, using response surface methodology it was possible to establish the fermentation conditions that maximize the total FOS production by *A. pullulans* (32 ºC, 385 rpm) and the validity of the model was proved by fitting the values of the variables in the model equation and carrying out the fermentation at those values of the variables. A 64,1 (±0,0) % ($g_{FOS}/g_{sucrose}$) total FOS production yield was obtained within 48 h of fermentation. Furthermore, it is important to notice that this production yield was obtained in a one-stage process, thus conferring this study an important role in the development of more efficient, less expensive processes for the industrial production of FOS.

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CHAPTER 4:
TRANSFRUCTOSYLATION ACTIVITY OF THE ENZYMES PRODUCED BY AUREOBASIDIUM PULLULANS



"Anyone who has never made a mistake has never tried anything new."

Albert Einstein

The separation and extraction of the transfructosylation enzymes from the extracellular, periplasmatic and intracellular spaces of *Aureobasidium pullulans*, as well as their transfructosylating activity (U_t) and hydrolyzing activity (U_h) ratio (U_t/U_h), is described in this chapter. This study demonstrated that the extracellular space of the enzyme(s) possess the higher transfructosylation activity.

4.1. INTRODUCTION

4.2. MATERIALS AND METHODS

4.2.1. Microorganism and culture conditions

4.2.2. Enzyme extraction procedure

4.2.3. Bright field microscopy

4.2.4. Enzymatic activity

4.2.5. Total protein determination – Microplate procedure

4.3. RESULTS

4.4. DISCUSSION

4.5. CONCLUSION

4.6. REFERENCES

4.1. INTRODUCTION

The production of fructooligosaccharides (FOS) has received particular attention in recent years because of their excellent biological and functional properties and demands the development of efficient enzymatic systems. Currently, FOS are produced commercially using either microbial FTase (EC 2.4.1.9) or FFase (EC 3.2.1.26) with high transfructosylation activity. For the industrial production of FOS, screening for microorganisms producing higher enzyme activities than the previously reported is of major importance, as it is the further information about the different types of FFases that exist and the optimum operational conditions for enzyme production (Hayashi *et al.* 1992a, Nguyen *et al.* 1999, L'Hocine *et al.* 2000).

Enzymes with transfructosylating activity act on sucrose by cleaving the β (1, 2) linkage and transferring the fructosyl group to an acceptor molecule such as sucrose, thus releasing glucose (Belghith *et al.* 2012). Although FFase is generally known as an enzyme catalyzing the hydrolysis of sucrose, some FFases have much higher transfructosylating activity (U_t) than hydrolyzing activity (U_h). A wide variety of fungi produce FFases, but the activity ratios (U_t/U_h) of their enzymes vary significantly (Sangeetha *et al.* 2005, Yoshikawa *et al.* 2006). Fructosyl-transferring enzymes have been purified and characterized from higher plants, such as asparagus (Shiomi 1982), onion (Fujishima *et al.* 2005), Jerusalem artichoke (Koops and Jonker 1994), and from different microorganisms (fungi and bacteria), such as *Aspergillus niger* (L'Hocine *et al.* 2000), *Aspergillus japonicus* (Hayashi *et al.* 1992), *Aureobasidium pullulans* (Yoshikawa *et al.* 2006), *Bacillus macerans* (Park *et al.* 2001) and *Candida utilis* (Chávez *et al.* 1997). Although these proteins differ in their subunit structure, molecular weight, degree of glycosylation, chemical susceptibility and substrate specificity, they all display both hydrolytic and transfer activities (Ghazi *et al.* 2007).

A. pullulans is of widespread ecological occurrence and it can occur in soil and plants. This fungus can grow well in the presence of a high concentration of sucrose, and it can produce polysaccharides, such as pullulan and β-glucan, oligosaccharides, such as FOS and isomaltooligosaccharides, and various carbohydrate-degrading enzymes (Deshpande *et al.* 1992, Yun *et al.* 1994, Hamada *et al.* 2000, Yoshikawa *et al.* 2006). Various studies indicate that *A. pullulans* produces multiple FFases, and that some of the enzymes have high transfructosylating activity. However, there have been few attempts to elucidate the total number of FFases produced by *A. pullulans*, or to compare their biochemical characteristics. Such information may help in the development of an efficient production process of FOS by *A. pullulans* (Jung *et al.* 1987, Hayashi *et al.* 1991, L'Hocine *et al.* 2000, Yoshikawa *et al.* 2006).

It remains uncertain why *A. pullulans* produces FOS from sucrose but this fungus is often isolated from sucrose-rich environments (e.g. fruits). If sucrose is hydrolyzed to glucose and fructose, it may cause much higher osmotic pressure in the environment because of the increased number of sugar molecules. On the other hand, the number of sugar molecules does not change in the

transformation of sucrose into FOS. Thus, FOS production by this fungus may be one of the defensive mechanisms against osmotic stress in sucrose-rich environments. Alternatively, FOS production might be advantageous to prevent competitive microorganisms from utilizing sucrose, as FOS is much less sensitive to microbial hydrolysis than sucrose (Yoshikawa *et al.* 2006).

The aim of this work is to extract the enzymes from the intracellular, periplasmatic and extracellular spaces of the fungi *A. pullulans* CCY 27-1-94 and to measure the activity ratio U_t/U_h of the FOS-producing enzymes produced by this microorganism in the different cellular spaces (intracellular, periplasmic and extracellular spaces).

4.2. MATERIALS AND METHODS

4.2.1. Microorganism and culture conditions

The fungus *A. pullulans* CCY 27-1-94 (Culture Collection of Yeasts, Bratislava, Slovakia) was grown according to the description made in the item 3.2.1. of this thesis. A volume of 300 mL of culture broth was collected from the reactor in the FOS maximum production point (48 h) under optimized fermentation conditions (T = 32 ºC, agitation speed = 385 rpm) and the procedure described in the following item was conducted in order to extract the enzymes from the different cellular spaces of *A. pullulans*.

4.2.2. Enzyme extraction procedure

The enzymes from the different cellular spaces of *A. pullulans* were extracted according to the procedure represented schematically in Figure 4.1. Detailed procedures related to each enzymatic fraction (extracellular, periplasmic and intracellular) are also presented in the next items.

Extracellular fraction

A volume of 100 mL of fermentation broth was added to a pre-weighted tube and the cells were harvested by centrifugation at 10.000 g x 10 min at 4 ºC. The pellet obtained was saved on ice until further use and the supernatant was dialyzed to remove the salts form the medium. The dialyzed sample was then lyophilized. Finally, the lyophilized sample was dissolved in 0,5 mL – 1 mL of ultra-pure water (minimum quantity to dissolve the sample) and stored at -20 ºC until enzymatic activity determination.

Periplasmic fraction

For the enzymes extraction from the periplasmic space, the pellet saved previously was ressuspended in in 5 mL of Tris-HCl 50 mM, pH 7,2 and 0,3 U.mgdry weight -1 of lyticase was added to the suspension. This suspension was then incubated for 2 h at 30 ºC. Lyticase, produced by a submerged culture of *Arthrobacter luteusis*, is an essential enzyme responsible for lysis of viable cell walls, mainly in yeast and acts by hydrolyzation of linear glucose polymers with β-1,3-linkages. After incubation, a sample to control the efficiency of the procedure, by microscope analysis (4.2.3), was removed. With the lyticase treatment the fungus cells are expected to turn thinner. Lastly, the suspension was centrifuged at 5.000 g for 15 min at 4 ºC and 1 mL of the supernatant was removed and stored at -20 ºC until enzymatic activity determination. The pellet was saved on ice until the extraction procedure of the enzymes from the intracellular fraction.

Intracellular fraction

On this procedure, the pellet saved on the enzymatic extraction of the periplasmic space was ressuspend in 2 mL of Tris-HCl 20 mM, pH 7,5. In order to lyse the cells by sonication, the ressupsended pelled was vortexed and sonicated on ice, with a microtip and with a power level between 4 – 5, at 40 % – 50 % duty for 15 – 20 bursts. It is important to maintain the sample on ice during all the process to avoid thermal denaturation of the proteins. After sonication a sample was taken in order to control the efficiency of the procedure by microscope analysis (4.2.3). With the sonication treatment the fungus cells are expected to be disrupted. A volume of 1,5 mL of sample was centrifuged for 10 min at 14.000 g. Finally, 0,5 mL of supernatant was removed and stored at -20 ºC until enzymatic activity determination.

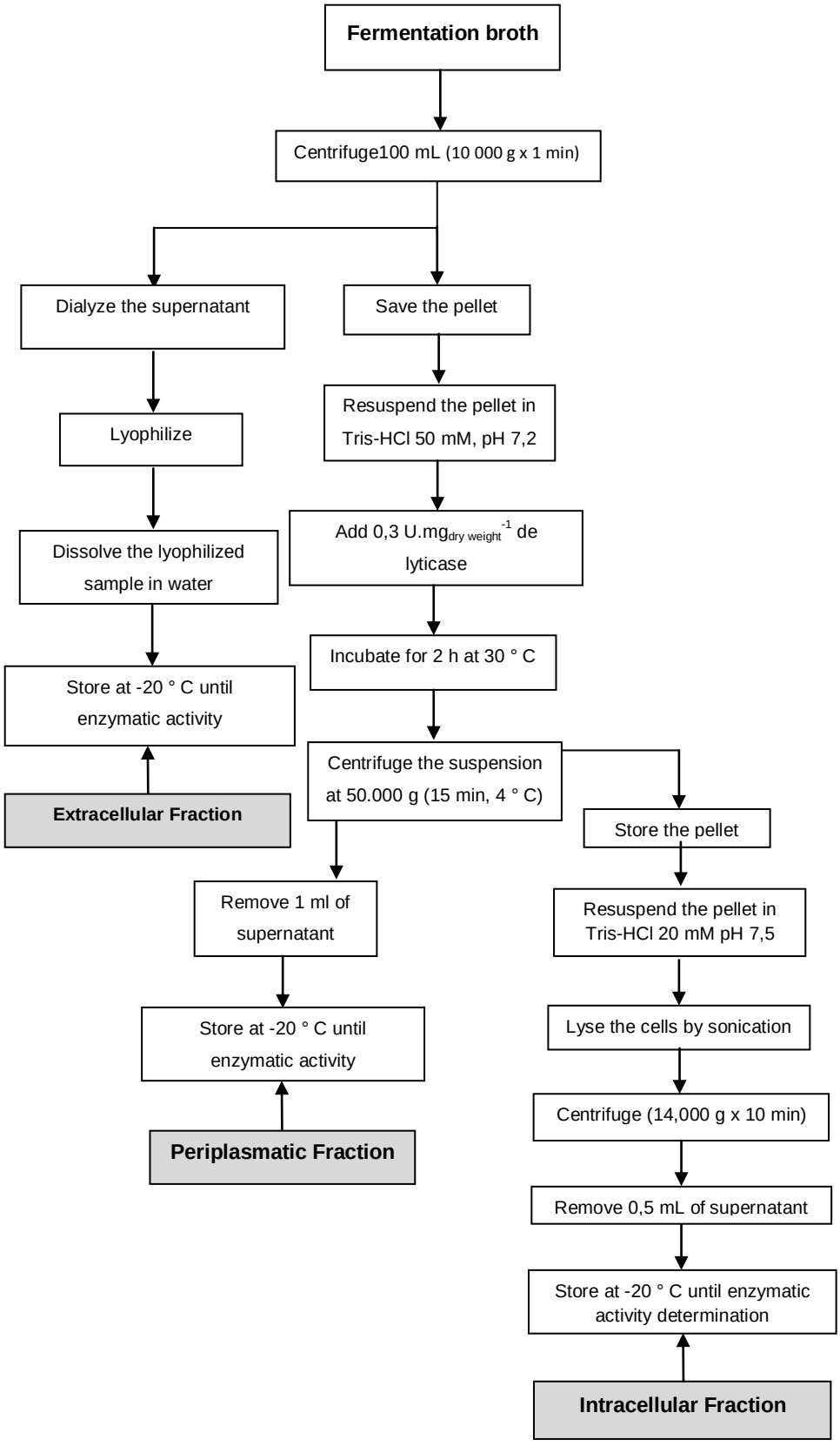


Figure 4.1. Schematic representation of the enzymatic extraction procedure of the different cellular spaces (extracellular, periplasmic and intracellular spaces) of *A. pullulans*.

4.2.3. Bright field microscopy

As mentioned previously, the enzyme extraction procedure was monitored by microscope analysis. Images were acquired with a Leitz Laborlux S optic microscope (Leitz), with 100 x magnification, coupled to a Zeiss Axio Cam (Zeiss). Image acquisition was performed by the software Axio Vision 3.1 (Zeiss).

4.2.4. Enzymatic activity

Transfructosylating activity (U_t) and hydrolyzing activity (U_h) were determined by the method described by Yoshikawa *et al.* (2006). Briefly, in the first step, FFase activity was determined by measuring the amount of glucose produced from sucrose. The reaction mixture contained 100 µL of the crude FFase extract, 300 µmol of sucrose and 50 µmol of sodium acetate buffer (pH 5.0) in a total volume of 1 mL. After incubation for 20 min at 30 ºC, the reaction was stopped by boiling. One unit (U) of the FFase activity was defined as the amount of enzyme that released 1 µmol of glucose per min. U_t and U_h activities were determined by measuring concentrations (mM) of kestose and fructose, respectively, produced from sucrose. The reaction was carried out for 180 min using 0,5 $U.mL^{-1}$ of FFase in the reaction mixture described. The sugars concentrations were measured by HPLC, according to the method described previously in 3.2.3.

4.2.5. Total protein determination – Microplate procedure

The total protein content was determined according to the Bradford method, with bovine serum albumin (BSA) as a standard, using Coomassie Plus - The Better Bradford™ Assay Kit from Pierce. Two calibration curves were made. For the calibration curve for low protein concentration detection (1 $mg.L^{-1}$ – 25 $mg.L^{-1}$), triplicate volumes of 1, 2,5, 5, 10, 20 and 25 µL of 1 $mg.mL^{-1}$ BSA standard solution were pipetted into the wells of an ELISA plate and the volume adjusted to 150 µL with distilled water. The blank standard was made with distilled water. A volume of 150 µL Coomassie reagent was then added and the plate agitated in the ELISA spectrophotometer for 30 s. For the most consistent results, the plate was incubated for 10 minutes at room temperature. The absorbance of the samples was read at 595 nm wave length.

The second calibration curve was made for protein determination within the ranges of 100 $mg.L^{-1}$ to 1.500 $mg.L^{-1}$. A volume of 10 µL of each standard was pipetted into the appropriate microplate wells and 300 µlL of the Coomassie Plus Reagent was added to each well. The plate was mixed with plate shaker for 30 seconds and incubated for 10 min at room temperature. The blank standard was made with distilled water. Once again, absorbance of the samples was read at 595 nm wave length in the ELISA spectrophotometer.

For total protein determination of the enzymatic fractions, the same procedures were applied to the unknown samples, depending on the expected total protein content and the calibration curve applied to each reading.

4.3. RESULTS

The enzyme extraction procedure described in 4.2.2 was applied to a sample of the fermentation broth collected in the maximum FOS production point. This procedure was monitored by microscope analysis, being the images of the fungus along the extraction procedure presented in Figure 4.1.

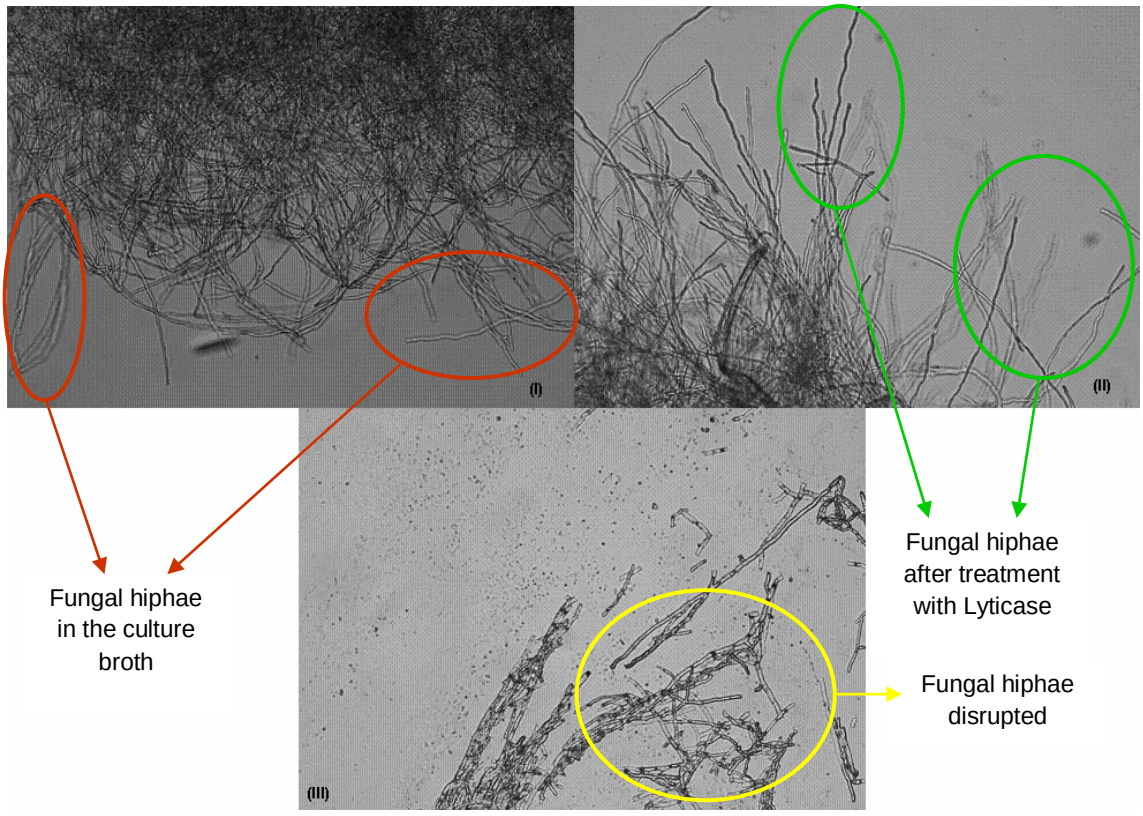


Figure 4.2. Microscope images of *A. pullulans* cells after enzyme extraction procedures with 100 x magnification (I – cells on the culture broth; II – cells after treatment with Lyticase; III – cells disrupted by sonication).

To evaluate the catalytic property of the FOS-producing enzymes in each cellular space (extracellular, periplasmatic and intracellular) the ratios of transfructosylating activity (U_t) to hydrolyzing activity (U_h) were determined. These results are gathered in Table 4.1.

Table 4.1. Enzymatic activity (hydrolytic activity - U_h and transfructosylation activity - U_t) and total protein of the enzymes extracted from the extracellular, periplasmatic and intracellular spaces of *A. pullulans*.

Fraction	U_h*	U_t*	Ratio U_t/U_h	Total Protein ($mg.L^{-1}$)
Extracellular	23,10 ± 0,00	22,71 ± 0,00	0,98 ± 0,00	362,19 ± 10,16
Periplasmatic	3,79 ± 0,00	0,70 ± 0,00	0,18 ± 0,00	24,94 ± 0,5
Intracellular	8,87 ± 0,00	10,50 ± 0,00	1,18 ± 0,00	1.264,58 ± 77,58

* Hydrolyzing activity (U_h) and transfructosylating activity (U_t) were determined by measuring concentrations (mM) of fructose and kestose formed by the enzyme reaction, respectively, by the HPLC method described in 3.2.3. The errors associated to these values are the ones associated to the HPLC method (Harvey 1956).

4.4. DISCUSSION

Some species of the genus *Aureobasidium* are known to produce various carbohydrate-degrading enzymes, such as pectinase, xylanase and glucosyltransferase (Deshpande *et al.* 1992, Yun *et al.* 1997). FFase is one of the carbohydrate-degrading enzymes produced by *Aureobasidium*, and catalyzes not only hydrolysis but also transfructosylation of sucrose to produce FOS, whereas FTase only catalyzes the sucrose transfructosylation reaction. However, there are still many uncertainties with regard to the diversity of enzymes with transfructosylation activity in *Aureobasidium* (Yoshikawa *et al.* 2006).

Although *A. pullulans* enzymes are used in the industrial production of FOS, there is much less information available about its properties compared with that from Aspergillus sp. Two intracellular and two extracellular FTases from *Aureobasidium sp.* ATCC 20524 were identified and characterized (Hayashi *et al.* 1991, Hayashi *et al.* 1992b). Lee *et al.* (1992) purified intracellular FTase from *A. pullulans* C-23 and described its properties. Antošová *et al.* (2002) identified FTase activity in *A. pullulans* CCY 27-1-1194 and studied the effect of sucrose concentration in the enzymatic activity. Later on, the authors reported FTase chromatographic separation and kinetic properties (Antošová *et al.* 2008). On 2004, Sangeetha *et al.* studied the production FOS by extracellular FTase from *A. pullulans* CFR 77. Yoshikawa *et al.* (2006) separated five types of FTase differing in the ratio of transfructosylation and hydrolyzing activity from the cell wall of *A. pullulans* DSM2404. In a subsequent publication, the enzyme with the highest transfructosylating activity was purified and its properties were investigated (Yoshikawa *et al.* 2007). Lateef *et al.* (2007a) studied the effect of ultrasonication on the release of FTase from *A. pullulans* CFR77 and later on (Lateef *et al.* 2007b) reported the purification and partial characterization of an intracellular FTase from *A. pullulans* CFR77.

In this study, to evaluate the efficiency of the enzymatic extraction, the procedure was monitored by microscope analysis. The effect of the lyticase treatment is only slightly visible by this analysis (Figure 4.1 – II). With the lyticase treatment it was expected for the fungus cells to turn thinner

and although this effect seems to be visible in some parts of the cells, this is not perfectly noticeable in the entire image. The disruption of the cells after sonication is clearly visible in Figure 4.1 – III, indicating that the enzymes from the intracellular space must have been released to the medium.

The higher transfructosylation activity was detected in the crude extract of the enzymes of the extracellular space of *A. pullulans*. These results are in accordance with Antošová *et al.* (2002) that reported transfructosylation activity in the same *A. pullulans* strain used in the present study, in the extracellular medium and in the whole cells. In their study, a maximum FTase transfructosylation activity of 131.000 $U.dm^{-3}$ (131 $U.mL^{-1}$) released into the cultivation medium, after 4 cultivation days, was reported. They also reported that with an initial sucrose concentration of 200 $g.L^{-1}$, an FTase transfructosylation activity of 80.000 $U.dm^{-3}$ (80 $U.mL^{-1}$) is achieved. This value is higher than the one obtained in present study (22,71 $U.mL^{-1}$) but this variation may be due to the differences between the transfructosylation activity determination methods.

On the other hand, it is in the intracellular space that the total protein content is higher as well as the ratio U_t/U_h. In 2008, Antošová *et al.* reported the chromatographic separation of an intracellular FTase from *A. pullulans*. The isolated enzyme exhibited both hydrolytic and transferase activity, being the transfructosylation activity higher in the sucrose concentration range tested (10 $g.L^{-1}$ – 600 $g.L^{-1}$) and completely dominating at higher sucrose concentrations, which is concordant to our findings.

The enzymes from the periplasmic space present the lower U_t, U_h and U_t/U_h ratio. Yoshikawa *et al.* (2006) have reported five FFase extracted from the cell wall of *A. pullulans* with higher U_t, U_h and U_t/U_h ratios than the ones obtained in the present study, which may be due to the different *A. pullulans* strains used in both researches, since both enzymatic extraction and activity determination procedures are the same. Nonetheless, in a later study Yoshikawa *et al.* (2007) reported FOS production by crude FFase preparation, reaching similar FOS production yields to the ones obtained in this study (Chapter 3).

It is important to notice that the fact that the higher transfructosylation activity of the *A.pullulans* strain used in this study was found in the extracellular medium supports the idea of developing a one stage process for the production of FOS.

4.5. CONCLUSION

Concluding, using the proposed enzyme extraction methodology it was possible to extract the enzymes of the extracellular, periplasmic and intracellular spaces. The higher transfructosylation activity (U_t = 22,71 $U.mL^{-1}$) was found for the enzyme(s) present in the extracellular space, thus supporting the idea of developing a one-stage FOS production process. On the other hand, the higher U_t/U_h ratio (1,18) was obtained for the enzyme(s) present in the intracellular space.

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CHAPTER 5:
FOS PRODUCTION BY FED-BATCH FERMENTATION OF SUCROSE BY AUREOBASIDIUM PULLULANS



"If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein.

In this chapter, FOS production by fed-batch fermentation of sucrose by *Aureobasidium pullulans* is described. With the main goal of increasing FOS production, comparing to the batch experiments, two feeding profiles (constant feeding and linear feeding) were tested. Using fed-batch fermentation by *A. pullulans* with a linear feeding profile, a slightly higher FOS production yield was achieved, as well as a higher concentration of fructofuranosyl nystose. Nevertheless, the batch productivity was found to be three times higher.

5.1. INTRODUCTION

5.2. MATERIALS AND METHODS

5.2.1. Microorganism and culture conditions

5.2.2. Fermentation Operation

5.2.3. Sugar analysis

5.2.4. Cell growth

5.2.5. Enzymatic activity

5.2.6. FOS production yield and productivity determination

5.3. RESULTS

5.3.1. "Fed-Batch 1" – Constant feeding profile

5.3.2. "Fed-Batch 2" – Linear feeding profile

5.4. DISCUSSION

5.5. CONCLUSION

5.6. REFERENCES

5.1. INTRODUCTION

The role of FOS in human nutrition is undoubtedly beneficial for health and many food companies worldwide use them as food ingredients in several products. Industrial production of FOS is based on the transformation of sucrose by enzymes with a transfructosylating activity and microorganisms such as *A. pullulans, A. nige*r and *A. japonicus* are the most important industrial sources of high activity FTase and/or FFase enzymes (Antošová *et al.* 2002).

Batch production of fungi transfructosylation enzymes has been widely reported (Yoshikawa *et al.* 2008, Mussato *et al.* 2009, Dominguez *et al.* 2012). Nevertheless, there are only a few studies regarding the FTase and/or FFase production using fed-batch regimens. Chen (1995) compared FFase production by *A. japonicus* in shaken flasks, batch and fed-batch cultures and found that fed-batch cultivation using intermittent sucrose supply produced more FFase as compared to the batch culture. Later on, Yun *et al.* (1997) reported enhanced FTase production from *A. pullulans* as a result of substrate-feeding experiments. With sucrose supply to the media, a significantly increase of both the final concentration and specific activity of FTase was achieved. More recently, Dhake and Patil (2007) observed enhanced FTase production by *P. purpurogenum* when sucrose content was restored by additional feeding of fresh sucrose solution to the cultivation medium during the production period.

Fed-batch fermentation is a production technique in between batch and continuous fermentation. Over the past few years, the fed-batch operation mode has been used in an increasing number of fermentation processes to prevent the accumulation of by-products (potential inhibitors), thus allowing high biomass densities within a short cultivation time. Fed-batch cultivation can overcome the issues of substrate inhibition by slow feeding of nutrients to the bioreactor. Also, by-product formation can be avoided in fed-batch mode due to the possibility of restricting the growth rate, thus also avoiding limitations in oxygen transfer and heat transfer. The optimal design of fed-batch cultivation has to take into account several factors as the time point to start feeding fresh nutrients; substrate concentration and rate of addition in the feed; and time point to finish the nutrient feeding, so that the highest concentration of product is achieved and no unconverted substrate remains when the reactor is full. It is rather difficult to do trial and error experimentation with so many “open ended” variables, which may play key roles in the overall performance of the fed-batch cultivation. On the other hand, it is easier to maintain sterility under fed-batch conditions as compared to continuous cultures (Strandberg and Enfors 1991, Rocha 2003). Aiming at an increase in productivity, fed-batch operation is used for the production of several industrial products such as antibiotics, like penicillin, *Saccharomyces cerevisiae* yeast, cellulose by *Trichoderma reesei* and recombinant protein, like the Hepatitis B surface antigen (HbsAg) used as a vaccine against type B hepatitis. In fact, fed-batch fermentation does not require any special piece of equipment in addition to the one usually required for batch fermentation. It is only characterized by a slight longer overall time cycle that is certainly acceptable in industrial practice where currently very effective operating procedures for sterilization and the prevention of

contamination are used (Gu *et al.* 1991, Longobardi 1994). As opposed to the batch fermentation, in fed-batch cultures it is possible to manipulate the specific growth rate at an appropriate value providing a desirable metabolic condition, resulting in maximum productivity (Rocha and Ferreira 2006).

In a fed-batch process, a basal medium supports initial growth and production, and a feed medium prevents depletion of nutrients and sustains the production phase. Those media can contain distinct combinations of nutrients to accommodate the distinct metabolic requirements during different production phases. Process parameter settings — including feeding strategy and control parameters — define the chemical and physical environments suitable for cell growth and protein production. Optimization of a fed-batch process can be achieved by development efforts addressing one or more of three major elements: basal medium, feed medium, and process settings. Given the large set of variables in these systems, establishing a cost and time-efficient approach for process optimization is desirable but challenging (Jiang *et al.* 2012). Significant improvement in product concentration and production yield /productivity is possible if the nutrient feeding is conducted during the exponential phase of the cell growth. The simplest feeding profile is constant feeding of suitably selected nutrient concentration. The advantage of constant feeding strategy is that it is very simple and it does not require computer coupled peristaltic pump to implement the feeding strategy. However, the disadvantage is that it may lead to substrate accumulation in the fermentation medium, which needs to be fermented, so that, in the end, no unconverted substrate is left in the bioreactor. On the other hand, in the linear feeding profile the nutrient feed is linearly or exponentially increased at predetermined time of cultivation. If the feeding rate coincides with the growth rate in the bioreactor, by model simulations, it may be possible to achieve non-limiting and non-inhibitory concentration of substrate during the feeding period. However, at the end of the feeding it may be necessary to conduct a second batch cultivation to enable the consumption of the residual substrate in the bioreactor (Stanbury *et al.* 1995).

The aim of this study is to increase FOS production using a fed-batch fermentation of sucrose by *A. pullulans*. Two feeding strategies (constant and linear feeding profiles) were conducted under the optimized fermentation conditions previously established for batch operation (T = 32 ºC and agitation speed = 385 rpm) (Chapter 3).

5.2. MATERIALS AND METHODS

5.2.1. Microorganism and culture conditions

The procedure herein conducted was similar to the one described in 3.2.1. The fungus *A. pullulans* CCY 27-1-94 (Culture Collection of Yeasts, Bratislava, Slovakia) was stored at -20 ºC in liquid medium with glycerol (17 % (v/v) (pre-inoculum)). The inoculum was prepared by transferring 10 mL of pre-inoculum into the inoculation medium and subsequently it was grown for 3 days at 28 ºC and 140 rpm. The inoculum (3 % (v/v)) was transferred to the fermentation medium containing sucrose (120 $g.L^{-1}$), $NaNO_3$ (12 $g.L^{-1}$), $FeSO_4.7H_2O$ (0,01 $g.L^{-1}$), K_2SO_4 (0,35 $g.L^{-1}$) and $MgSO_4.7H_2O$ (0,5 $g.L^{-1}$). The fermentation was carried under the optimized temperature and agitation conditions determined for batch operation as described in the Chapter 3 (T = 32 ºC and agitation speed = 385 rpm).

5.2.2. Fermentation Operation

Two fed-batch fermentations were conducted in a 5 L Biostat MD fermenter from B. Braun Biotech (Germany) connected to a digital control unit, under two different feeding profiles. In “Fed-Batch 1”, a constant feeding profile was applied by feeding a sucrose solution with a concentration of 400 $g.L^{-1}$, at a feeding rate of 15 $mL.h^{-1}$, after an initial batch phase with an initial fermentation volume of 1 L, that was conducted for 36 h at T = 32 ºC and agitation speed = 385 rpm. “Fed-Batch 2” was conducted with a linear feed profile, where a sucrose solution with a concentration of 400 $g.L^{-1}$ was fed to the fermenter. The linear feeding started with an initial feeding rate of 5 $mL.h^{-1}$ that was increased linearly every 3 min, after an initial batch phase with an initial fermentation volume of 1,5L, that was conducted for 56 h at T = 32 ºC and agitation speed = 385 rpm.

The feeding strategy chosen was determined by a dynamical model for the fermentative production of fructooligosaccharides developed by Rocha (2009). The model accounts for hydrolysis and transfructosylation reactions, as well as biomass formation and it contains 27 parameters that were determined from experimental data using a Systems Biology toolbox with the Simulated Annealing method for curve fitting, with the global aim to optimize the feeding profile to maximize FOS production (Rocha *et al.* 2009).

5.2.3. Sugar analysis

Sugar concentrations in the fermentation broth were analyzed by high-performance liquid chromatography (HPLC), as previously described in 3.2.3. A modular liquid chromatograph (Jasco) equipped with a Prevail Carbohydrate ES column (5 µm, 250 x 4,6 mm) from Alltech was used, at room temperature. Elution was achieved using a mixture of acetonitrile and 0,04 % ammonium hydroxide in water (70:30 v/v) at a flow rate of 1,0 $mL.min^{-1}$. The response of the refractive index detector was recorded and integrated using the Star Chromatography Workstation software (Varian) (Dias *et al.* 2009).

5.2.4. Cell growth

Cellular growth was quantified by biomass determination (dry weight) using a microwave oven, as previously described in 3.2.4. Briefly, 10 mL of of the fermentation broth was filtered through a 0,45 µm filter and dried in a microwave oven for 15 min at 150 W. The filter was then placed in a desiccator to cool for 10-15 min and afterwards it was weighted. Also, all filters were dried and weighted before the filtration (Olsson and Nielsen 1997). This procedure was done in triplicate for each fermentation broth sample.

5.2.5. Enzymatic activity

Transfructosylating activity (U_t) and hydrolyzing activity (U_h) were determined by the method previously described in 4.2.4. (Yoshikawa *et al.* 2006). One unit (U) of the FFase activity was defined as the amount of enzyme that released 1 µmol of glucose per min. U_t and U_h activities were determined by measuring concentrations (mM) of kestose and fructose produced from sucrose, respectively. The sugars concentrations were measured by HPLC according to the method described in 3.2.3.

5.2.6. FOS production yield and productivity determination

FOS production yield in the fed-batch experiments were determined according to the following equation:

$$Y_P\ (g_{FOS}.g_{sucrose}^{-1}) = \frac{V_f \times P_f - V_i \times P_i}{F \times \theta \times S_0 + V_i \times S_i - V_f \times S_f} \quad \text{(Equation 5.1)}$$

were Y_P is the FOS production yield ($g_{FOS}.g_{sucrose}^{-1}$), V_i and V_f the volume of the fermentation medium (L) in the beginning and at the end of the fermentation, respectively, P_i and P_f the FOS concentration measured in the aqueous phase ($g.L^{-1}$) in the beginning and at the end of the fermentation, respectively, F the sucrose feeding rate ($L.h^{-1}$), θ the duration of the fermenter feeding phase (h) and S_i and S_f the sucrose concentration measured in the aqueous phase ($g.L^{-1}$) in the beginning and at the end of the fermentation, respectively.

For the process productivities determination, the equation 5.2 was applied.

$$Q\ (g_{FOS}.L^{-1}.h^{-1}) = \frac{V_f \times P_f - V_i \times P_i}{V_f \times t_f} \quad \text{(Equation 5.2)}$$

where Q is the process productivity ($g.L^{-1}.h^{-1}$), V_i and V_f the volume of the fermentation medium (L) in the beginning and at the end of the fermentation, respectively, P_i and P_f the FOS concentration measured in the aqueous phase ($g.L^{-1}$) in the beginning and at the end of the fermentation, respectively and t the fermentation time (h).

5.3. RESULTS

5.3.1. “Fed-Batch 1” – Constant feeding profile

Fed-batch fermentation of sucrose by *A. pullulans* was conducted for 120 h. In order to eliminate the inhibitory effect of glucose accumulation in the fermentation broth, the initial sucrose concentration in the culture media was set to be 100 g. L^{-1}. After an initial batch phase (≈36 h), a sucrose solution (400 $g.L^{-1}$) was fed to the fermenter at a constant flow rate of 15 $mL.h^{-1}$. Fermentation samples were taken for sugar concentration, cellular growth and transfructosylation and hydrolyzing activities determination, being these results presented in Figures 5.1., 5.2. and 5.3. The FOS production yield and productivity of this experiment are given in Table 5.1.

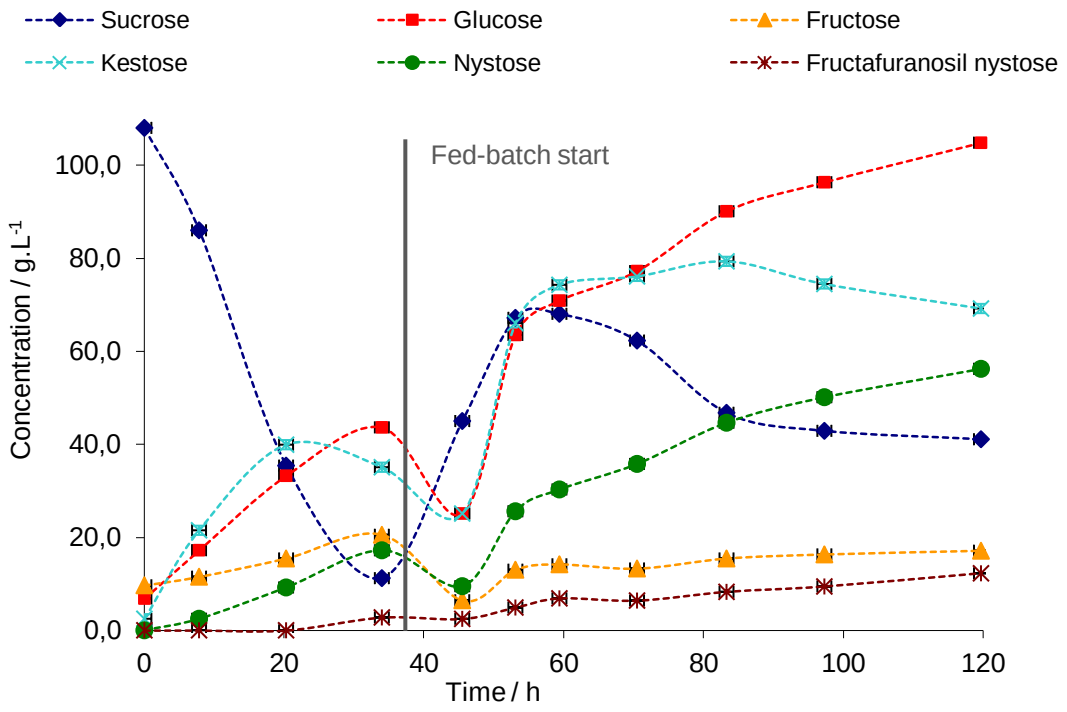


Figure 5.1. Fed-batch fermentation of sucrose by *A. pullulans* at constant feeding profile: kinetics of sucrose consumption and glucose, fructose, kestose, nystose and fructofuranosyl nystose production (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method.

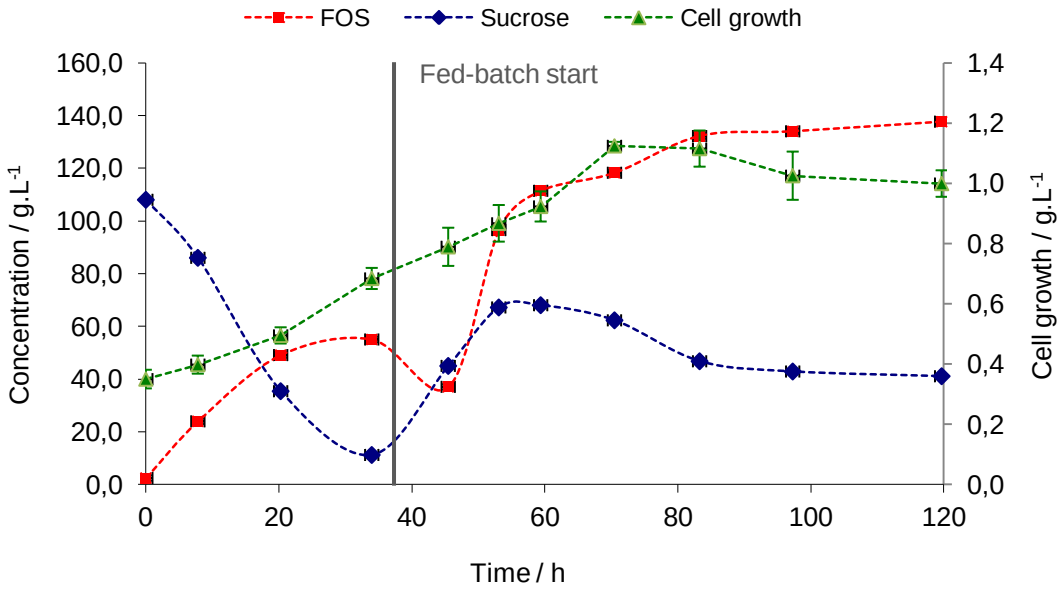


Figure 5.2. Fed-batch fermentation of sucrose by *A. pullulans* at constant feeding profile: kinetics of cell growth (biomass concentration/g.L^{-1}), total FOS production and sucrose consumption (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method.

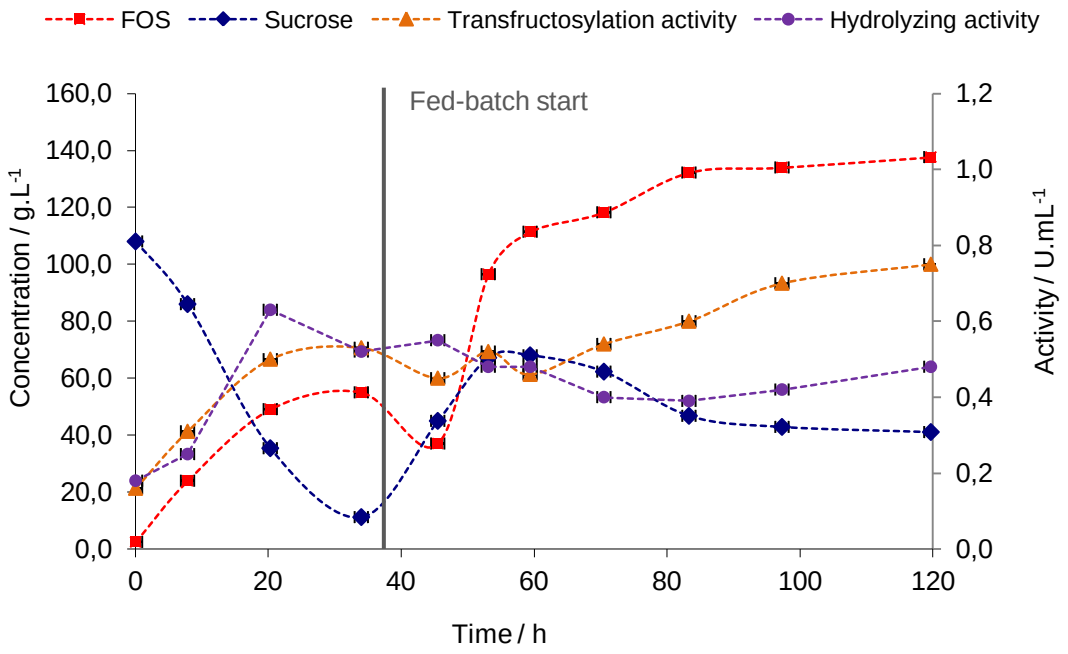


Figure 5.3. Fed-batch fermentation of sucrose by *A. pullulans* at constant feeding profile: Kinetics of total FOS production, sucrose consumption (concentration/g.L^{-1}) and transfructosylation and hydrolyzing activity (activity/U.mL^{-1}). The errors associated to sugar concentrations and enzymatic activity are the ones associated to the HPLC method.

Table 5.1. FOS production yield (Y_P,% ($g_{FOS}.g_{sucrose}^{-1}$)) and productivity (Q, $g.L^{-1}.h^{-1}$) in "Fed-Batch 1" – constant feeding profile, "Fed-Batch 2" – linear feeding profile and Batch optimized experiment (data from Chapter 3).

	Y_P (% ($g_{FOS}.g_{sucrose}^{-1}$))	Q ($g.L^{-1}.h^{-1}$)
Fed-Batch 1	60,0 ± 0,0	1,15 ± 0,00
Fed-Batch 2	65,4 ± 0,0	0,85 ± 0,00
Batch	64,1 ± 0,0	2,67 ± 0,05

5.3.2. "Fed-Batch 2" – Linear feeding profile

In "Fed-batch 2", a fed-batch fermentation of sucrose by *A. pullulans* was conducted for 170 h, in the same conditions as the ones described for "Fed-Batch 1". After an initial batch phase (≈56 h), a sucrose solution (400 $g.L^{-1}$) was fed to the fermenter at flow rate of 5 $mL.h^{-1}$ that was linearly increased every 3 min. Similarly as in 5.3.1., fermentation samples were taken for sugar concentration, cellular growth and transfructosylation and hydrolyzing activities determination, being these results presented in Figures 5.4., 5.5. and 5.6. The FOS production yield and productivity of this experiment is given in Table 5.1.

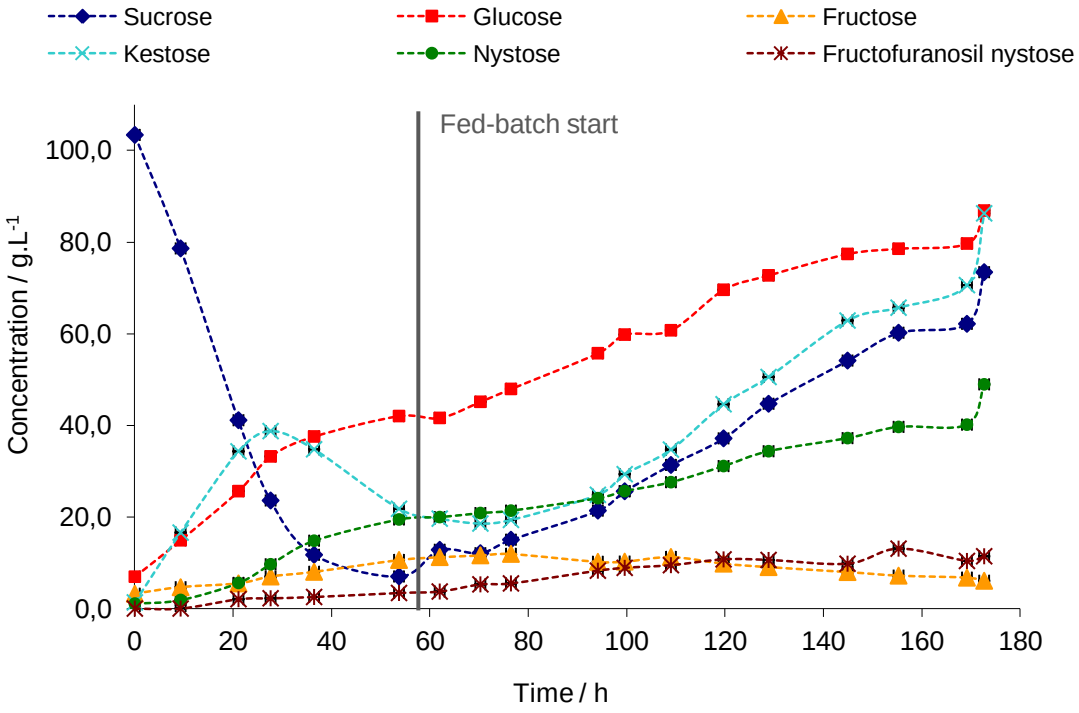


Figure 5.4. Fed-batch fermentation of sucrose by *A. pullulans* at linear feeding profile: kinetics of sucrose consumption and glucose, fructose, kestose, nystose and fructofuranosyl nystose production (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method.

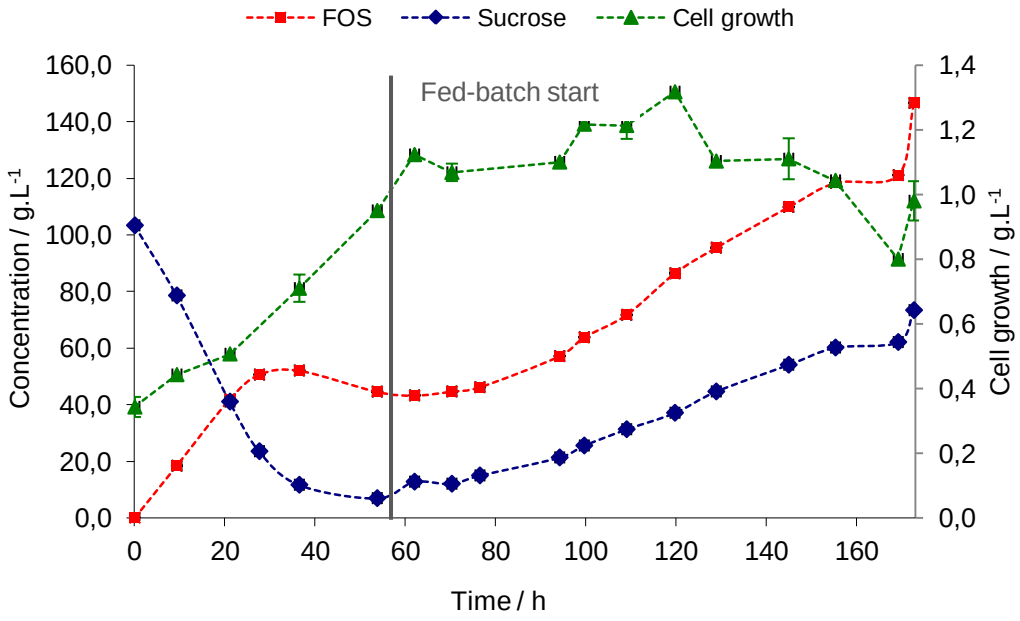


Figure 5.5. Fed-batch fermentation of sucrose by *A. pullulans* at linear feeding profile: kinetics of cell growth (biomass concentration /g.L^{-1}), total FOS production and sucrose consumption (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method.

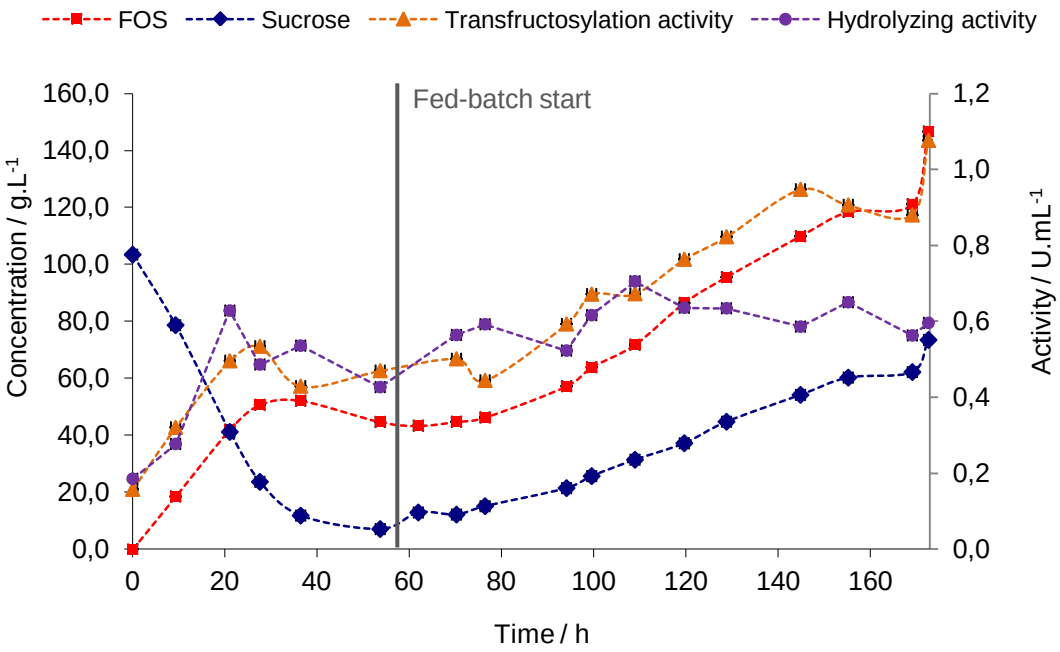


Figure 5.6. Fed-batch fermentation of sucrose by *A. pullulans* at linear feeding profile: kinetics of total FOS production, sucrose consumption (concentration/g.L^{-1}) and transfructosylation and hydrolyzing activity in the fermentation broth (activity/U.mL^{-1}). The errors associated to sugar concentrations and enzymatic activity are the ones associated to the HPLC method.

5.4. DISCUSSION

In the last two decades, many studies have investigated the production of oligosaccharides as food ingredients that promote a state of wellbeing and health of the consumers (Saad *et al.* 2013). In a fermentative process for FOS production, the substrate consumption for biomass growth must also to be considered, thus increasing even further the complexity of the process and motivating the application of mathematical modeling approaches such that non-obvious operation conditions can subsequently be found that maximize the productivity of FOS and minimize the accumulation of monosaccharides, mainly glucose (Rocha *et al.* 2009).

In Fed-Batch 1, with sucrose constant feeding rate to the fermentation broth, an increase in FOS production was observed up to 80 h of fermentation. From this time and up to 120 h of fermentation, FOS concentration was nearly constant (≈ 135 g.L^{-1}), suggesting that sucrose feeding may be insufficient. This suggestion is reinforced by the observation of kestose decrease during that period. Antošová *et al.* (2002) observed the effect of sucrose concentration on FTase activity, concluding that low sucrose concentration in the fermentation medium (50 g.L^{-1}) decreases FTase acivity and may even stop FTase production after two days. FOS production

yield by fed-batch fermentation with sucrose constant feeding was found to be lower and process productivity is more than half, when compared to the batch experiment.

With a linear feeding profile (Fed-Batch 2), a gradual increase of FOS production was found until the end of the fermentation period, reaching 147 g. L^{-1} after 173 h, accomplishing a FOS production yield slightly higher, when compared to both fed-batch experiment with a constant feeding and also to the batch experiment. Nonetheless, FOS productivity in linear feeding profile fed-batch fermentation was found to be more than two times lower when compared to the same experiments. Yet, it is important to take into account that the feeding initial rate of the linear profile was three times lower than the one used in the constant feeding experiment. The linear feeding profile seems to be more suitable for this production process. However, in the initial phase, the flow rate of 5 $mL.h^{-1}$ does not seem to be enough for maximizing FOS productivity.

Glucose, as a result of both hydrolyzing and transfructosylation activity of the FOS-producing enzymes is continuously accumulated in the fermentation media, as has occurred in the batch experiments. In these fed-batch experiments, glucose accumulation at the end of the fermentation (± 100 $g.L^{-1}$) is even higher than the one verified in the batch experiment (± 77 $g.L^{-1}$).

The main advantage of operating in fed-batch mode is that under these conditions no consumption of FOS was found contrarily to what has been observed for the batch operation mode, thus leading to a higher final concentration of FOS. Furthermore, it is also worth to notice that fructofuranosyl nystose (GF4) production was observed using the fed-batch operation mode. In the maximum total FOS production point for a batch operation mode, the FOS composition was found to be approximately 68 % 1-kestose and 32 % nystose. For the same final total FOS concentration, its composition obtained by fed-batch production was found to be approximately 58 % 1-kestose (GF2), 33 % nystose (GF3) and 9 % fructofuranosyl nystose (GF4). The presence of GF4 in the mixture can bring added value to the product since long-chain prebiotics may exert a prebiotic effect in more distal colonic regions compared with the lower-molecular-weight FOS, which may be more quickly fermented in the saccharolytic proximal bowel (Manning and Gibson 2004). Another difference observed in the fed-batch process was related to the fungus morphology. In the beginning of the fermentation, *A. pullulans* was in its pellet form and as the feeding occurred its morphology changed to the filamentous form. This observation may explain why an apparent reduction in the total biomass was found in the end of the fermentation (Figures 5.2 and 5.5), since it is quite difficult to sample biomass at this time points due to an increase of the fermentation broth density.

The main disadvantage of the fed-batch operation is related to the process productivity. Based on the results herein reported it was found that the FOS productivity in batch operation mode is three times higher comparing to the fed-batch operation mode using a linear feeding profile.

There are only a few studies regarding the FTase and/or FFase production using fed-batch regimens but, all of them report enhanced transfructosylation enzyme(s) production as a result of fed-batch substrate culture. Chen (1995) found that fed-batch cultivation of *A. japonicus*, using

intermittent sucrose supply at 26,6 % (w/v), produced more FFase than batch culture, and the maximal enzyme production was about 20 % higher. Similar effects were observed by Yun *et al*. (1997). With sucrose supply to the media, at a concentration of 280 g/L, a significantly increase of both the final concentration and specific activity of FTase was achieved, which were about 25 % - 47 % higher than in batch culture. More recently, Dhake and Patil (2007) observed enhanced FTase production by P. purpurogenum when sucrose content was restored by additional feeding of fresh sucrose solution at 1% (w/v) to the cultivation medium, during production period.

In our experiments and with the feeding profiles used, U_t and U_h activities were found to be around the same for both fed-batch and batch operation modes, with a little increase in U_t towards the end of "Fed-Batch 2" (linear feeding profile). Taking into consideration the dilution factor in the enzymatic fraction extraction procedure (Chapter 4), the U_t and U_h activities in the fermentation broth, for the maximum FOS production point in the batch experiment, were found to be near 0,6 $U.mL^{-1}$ ($U_t/U_h \approx 1,0$). By fed-batch operation with a linear feeding profile, a U_t/U_h ratio of 1,81 was obtained in the maximum FOS production point, which represents an increase in the ratio U_t/U_h, compared to the batch experiment.

5.5. CONCLUSION

In summary, with fed-batch fermentation of sucrose supplied by a linear feeding profile, using *A. pullulans*, a slightly higher FOS production yield can be achieved as compared to the batch production process. Nevertheless, the productivity in fed-batch operation mode was found to be three times lower than in batch mode. However, it is important to notice that with the fed-batch operation mode, fructofuranosyl nystose (GF4) was produced (this was not found for the batch operation mode), which can add more commercial interest to the FOS mixture produced since the GF4 may exert a prebiotic effect in more distal colonic regions compared with the lower-molecular-weight FOS.

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CHAPTER 6:
SCALE-UP OF A ONE-STAGE FOS PRODUCTION PROCESS



"Learning never exhausts the mind."

Leonardo da Vinci.

The scale-up of the one-stage process for FOS production from sucrose by *Aureobasidium pullulans* to a semi-pilot scale (75 L bioreactor), under optimized fermentation conditions, is described in this chapter. A scale increase in the production process of 25 times was applied and a 66,5 %($g_{FOS}/g_{sucrose}$) production yield and process productivity of 3,10 ± 0,00 g_{FOS}/L.h , were obtained after 48 h of fermentation. Moreover, the design and economics of an industrial unit for 10.000 ton/year of FOS production is presented.

6.1. INTRODUCTION

6.2. MATERIALS AND METHODS

6.2.1. Microorganism and culture conditions

6.2.2. Fermentation Operation

6.2.3. Sugar analysis

6.2.4. Cell growth

6.2.5. Process design and economics

6.3. RESULTS

6.3.1. FOS production on a semi-pilot scale

6.3.2. FOS industrial production design and economics

6.4. DISCUSSION

6.5. CONCLUSION

6.6. REFERENCES

6.1. INTRODUCTION

Currently, consumers believe that food directly interferes and contributes to their health. This increasing consumer awareness combined with the advances in various scientific domains provides unique opportunities for the food industry to come up with a number of new concepts in functional food development. Research has been primarily concerned with establishing the scientific bases in health, nutrition and food processing. Whatever the source, the ultimate market success depends on the extent to which the new product is able to meet the consumer needs (Sangeetha *et al.* 2005, Mussato and Teixeira 2010).

The commercial production of FOS is dated from 1984, under the name Meioligo, and was accomplished by Meiji Seika Co., using transfructosylation enzymes produced by *Aspergilus niger* (Vaňková *et al.* 2008). The theoretical yield ($g_{FOS}/g_{sucrose}$) of FOS from sucrose is 75 %, considering that 1-kestose is the only FOS being produced (Yoshikawa *et al.* 2008). However, the actual FOS yields reported are considerably lower, i.e., 56 % - 61 % using *A. japonicus* (Chiang *et al.* 1997, Wang and Zhou 2006) and 53 % – 59 % using *A. pullulans* (Yun *et al.* 1993, Shin *et al.* 2004). The overall stoichiometry of FTase and FFase actions on sucrose can be characterized by two parallel reaction paths (Jung *et al.* 1989). A set of disproportionate reactions provides FOS and glucose (as a by-product) from the transfructosylation activity of FTase, whereas the enzyme's hydrolytic activity results in the formation of glucose and fructose as by-products (Vaňková *et al.* 2008). Thus, the search for new potent producers of enzymes showing transfructosylating activity and the study of optimal conditions for transfructosylation are desirable in order to obtain the highest possible yield of good-grade FOS for potential industrial application and further commercialization (Fernández *et al.* 2004).

Despite the central role of the scale-up issue in biotechnology, and the comparably large body of literature, there seems to be no common, generally applicable strategy established. To increase product yields and to ensure consistent product quality, key issues of industrial fermentations, process optimization and scale-up are aimed at maintaining optimum and homogenous reaction conditions, minimizing microbial stress exposure and enhancing metabolic accuracy. The change in the geometry and physical conditions in larger scales, however, lead to a less favorable mixing behavior and to impaired physiological reaction conditions which, in turn, may lead to a decreased process constance and reproducibility, to reduced specific yields and to an increase in unwanted side products and thus ultimately to a diminished batch-to-batch consistency and product quality. Therefore, for each individual product, process and facility, a suitable scale-up strategy has to be elaborated by a comprehensive and detailed process characterization, identification of the most relevant process parameters influencing product yield and quality and their establishment as scale-up parameters to be kept constant as far as possible (Schmidt 2005).

Only a few studies have been reported about the scale-up effects on transfructosylation enzymes, and subsequently on the FOS production. Wang and Rakshit (1999) compared the FTase production by *A. faetidus* in flasks and different sized reactors, reaching a maximum fermenter

scale of 10 L with a 3,5 L working volume. Regarding studies on the scale-up and economic feasibility, a batch production process of purified FTase from *A. pullulans*, as well as a process for industrial production of FOS based on the conversion of sucrose by immobilized FTase produced by *A. pullulans* were designed by Vaňková *et al.* (2005, 2008), respectively. The FTase production plant was designed for an annual production capacity of 80 kg enzyme powder with a specific FTase activity of 1.143.000 U/g and the final mixture contained 28.4% FTase. The total capital investment estimated was 11,4 million Euros and an FTase price of about 200 Euros/g was recommended to obtain a payback time of 2 years. On the other hand, the FOS production plant was designed taking into account two alternative process flow sheets for an annual production of 10.000 tons of FOS, one for a powdery product and the second one for syrup. FTase produced by *A. pullulans* in the first stage of the process was immobilized and its activity was 40.000 $U.L^{-1}$, with an immobilization yield of 65 %. Under the conditions studied, the authors reported 89 % of sucrose conversion, and an annual production of syrup of 23.600 ton and of an alternative product of 10.000 ton (obtained by spray drying of the syrup).

Bearing in mind the importance of the scale-up effects in fermentation processes, the aim of this work is to scale up 25 times the optimized one stage process (described in Chapter 3) and analyze its effect on the FOS production. Moreover, based on laboratory and semi-pilot experiments, the design, feasibility and economics of an industrial FOS production process, for an annual output of 10.000 ton, was developed.

6.2. MATERIALS AND METHODS

6.2.1. Microorganism and culture conditions

The procedure herein conducted was similar to the one reported in 3.2.1. The fungus *A. pullulans* CCY 27-1-94 (Culture Collection of Yeasts, Bratislava, Slovakia) was stored at -20 ºC in liquid medium with glycerol (17 % (v/v) (pre-inoculum)). The inoculum was prepared by transferring 10 mL of pre-inoculum into the inoculation medium and subsequently it was grown for 3 days at 28 ºC and 140 rpm. The inoculum (3 % (v/v)) was transferred to the pre-fermentation medium containing sucrose (200 $g.L^{-1}$), $NaNO_3$ (20 $g.L^{-1}$), $FeSO_4.7H_2O$ (0,01 $g.L^{-1}$), K_2SO_4 (0,35 $g.L^{-1}$), KCl (0,5 $g.L^{-1}$), KH_2PO_4 (5 $g.L^{-1}$) and $MgSO_4.7H_2O$ (0,5 $g.L^{-1}$). The pre-fermentation was carried under the optimized temperature and agitation conditions described in the Chapter 3 (T = 32 ºC and agitation speed = 385 rpm) during three days. Afterwards, the fermentation broth was transferred to a 75 L fermenter using a 40 L working volume containing the same composition of the pre-fermentation medium.

6.2.2. Fermentation Operation

A semi-pilot scale fermentation was carried in a 75 L Laboratory Pilot Fermenter from Bioengineering (Switzerland) with a 40 L of working volume (Figure 6.1). The fermentation was conducted under the optimized fermentation conditions described in the Chapter 3 (T = 32 ºC and agitation speed = 385 rpm) during 108 h.



Figure 6.1. Laboratory Pilot Fermenter from Bioengineering (Switzerland) used in the semi-pilot scale 75 L experiment.

6.2.3. Sugar analysis

Sugar concentrations in the fermentation broth were analyzed by high-performance liquid chromatography (HPLC), as previously described in 3.2.3. A modular liquid chromatograph (Jasco) equipped with a Prevail Carbohydrate ES column (5 µm, 250 x 4,6 mm) from Alltech was used, at room temperature. Elution was achieved using a mixture of acetonitrile and 0,04 % ammonium hydroxide in water (70:30 v/v) at a flow rate of 1,0 $mL.min^{-1}$. The response of the refractive index detector was recorded and integrated using the Star Chromatography Workstation software (Varian) (Dias *et al.* 2009).

6.2.4. Cell growth

Cellular growth was quantified by biomass determination (dry weight) using a microwave oven, as previously described in 3.2.4. Briefly, 10 mL of of the fermentation broth was filtered through a 0,45 μm filter and dried in a microwave oven for 15 min at 150 W. The filter was then placed in a desiccator to cool for 10-15 min and afterwards it was weighted. Also, all filters were dried and weighted before the filtration (Olsson and Nielsen 1997). This procedure was done in triplicate for each fermentation broth sample.

6.2.5. Process design and economics

The process design was developed in the software SuperPro-Designer (Intelligen Inc., USA) and the production process is divided in two sections. In the first section, FOS are produced by one-stage fermentation of sucrose by *A. pullulans* under optimized fermentation conditions (Domiguez *et al.* 2012). The second section concerns to FOS purification from the fermentation broth using an activated charcoal column (Nobre *et al.* 2012). The economic analysis of the process design was performed using SuperPro-Designer and Microsoft Excel.

6.3. RESULTS

6.3.1. FOS production on a semi-pilot scale

To validate the one-stage FOS production process developed and optimized in this thesis, the process was scaled-up 25 times. On this experiment, an average total FOS yield of 66,5 ± 0,0 % ($g_{FOS}/g_{sucrose}$) and a process productivity of 3,10 ± 0,00 g_{FOS}/L.h were obtained after 48 h of fermentation, being the kinetics of sugar production and cell growth presented on Figures 6.1 and 6.2.

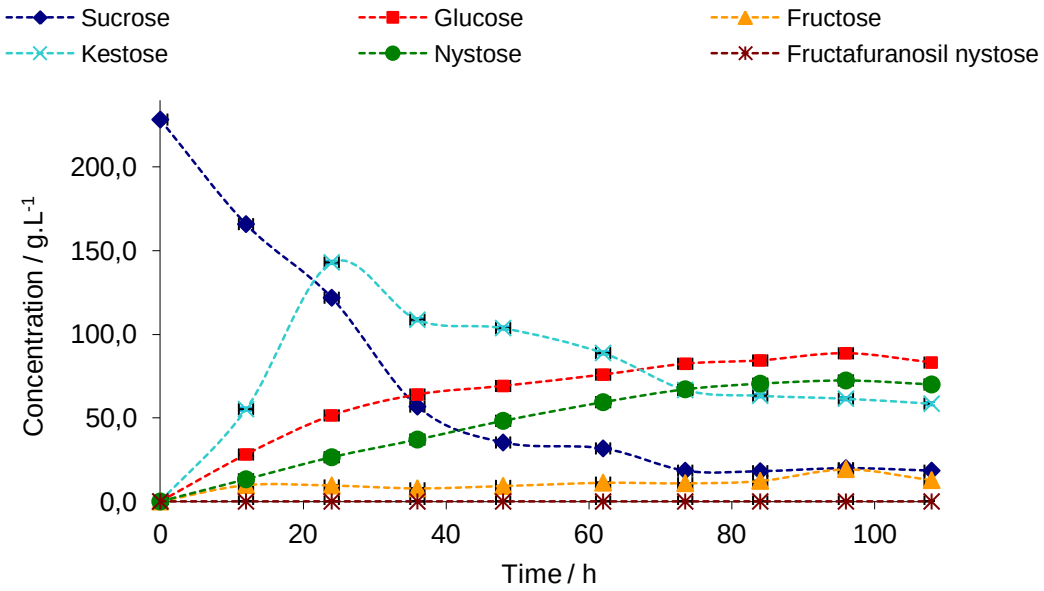


Figure 6.2. Semi-pilot scale one-step batch fermentation of sucrose by *A. pullulans*: kinetics of sucrose consumption and glucose, fructose, kestose, nystose and fructofuranosyl nystose production (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method.

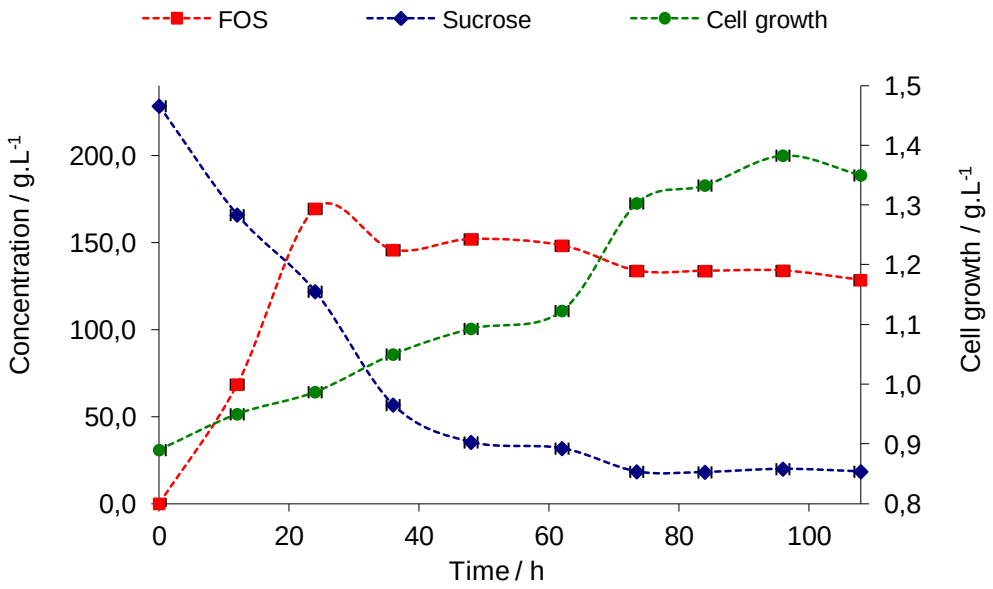


Figure 6.3. Semi-pilot scale one-step batch fermentation of sucrose by *A. pullulans*: kinetics of cell growth (cell growth/g.L^{-1}), total FOS production and sucrose consumption (concentration/g.L^{-1}). The errors associated to sugar concentrations are the ones associated to the HPLC method

6.3.2. FOS industrial production design and economics

Process Design

The process flowsheet was implemented in SuperPro-Designer software and is presented in Figure 6.4., consisting in two main sections, FOS production from sucrose fermentation by *A. pullulans* and FOS purification from the fermentation broth. This design was developed based on results obtained at lab-scale, as well as semi-pilot experiments.

In this process project, the fermentation procedure is accomplished in two sets of batch experiments, “Batch 1” and “Batch 2”, which are held offset to ensure a constant fermentation flow to the FOS purification step. In each step, FOS production is fulfilled in two fermenters (“Batch 1” – P-3/FR-103 and P-4/FR-104, “Batch 2” – P-5/FR-105 and P-6/FR 106) operating in parallel. This design was chosen in order to have each fermenter for FOS production with a volume of 250 m^3. In these batch operations, a cultivation medium containing sucrose at 100 $g.L^{-1}$, salts solution and 3 % inoculum are prepared in the 20 m^3 reactors P-1/FR-101 and P-2/FR-102. The inoculum prepared are then transferred to the fermenters for FOS production, which have a cultivation medium containing sucrose at 200 $g.L^{-1}$, salts solution and 3 % inoculum. At the end of the fermentation, the content of the fermenters is transferred to the decantation tank (P-14/V-101) to grossly separate the biomass from the fermentation broth, and thereafter into a buffer tank (P-9/V-102). The FOS purification section begins with the microfiltration (P-13/MF-101) of the broth, using a 0,2 µm filter pore size, to remove all the biomass from the broth. Residual proteins are then removed with a centrifugal filter (P-8/CX-101). Cell and protein-free broth is then stored in a second buffer tank (P-10/V-103) before FOS purification in activated charcoal columns (P-16/GAC-101). The product after purification has a purity of 93 %, being recovered 74,5 % (w/w) of the FOS obtained in the fermentation processes. A gradient of ethanol is used to recover the retained sugars in the charcoal. The sugars collected in the desorption phase are then subjected to an evaporation procedure, to remove ethanol, and subsequently are dried, in the spray dryer (P-15/SDR-10), to obtain the final FOS powder product.

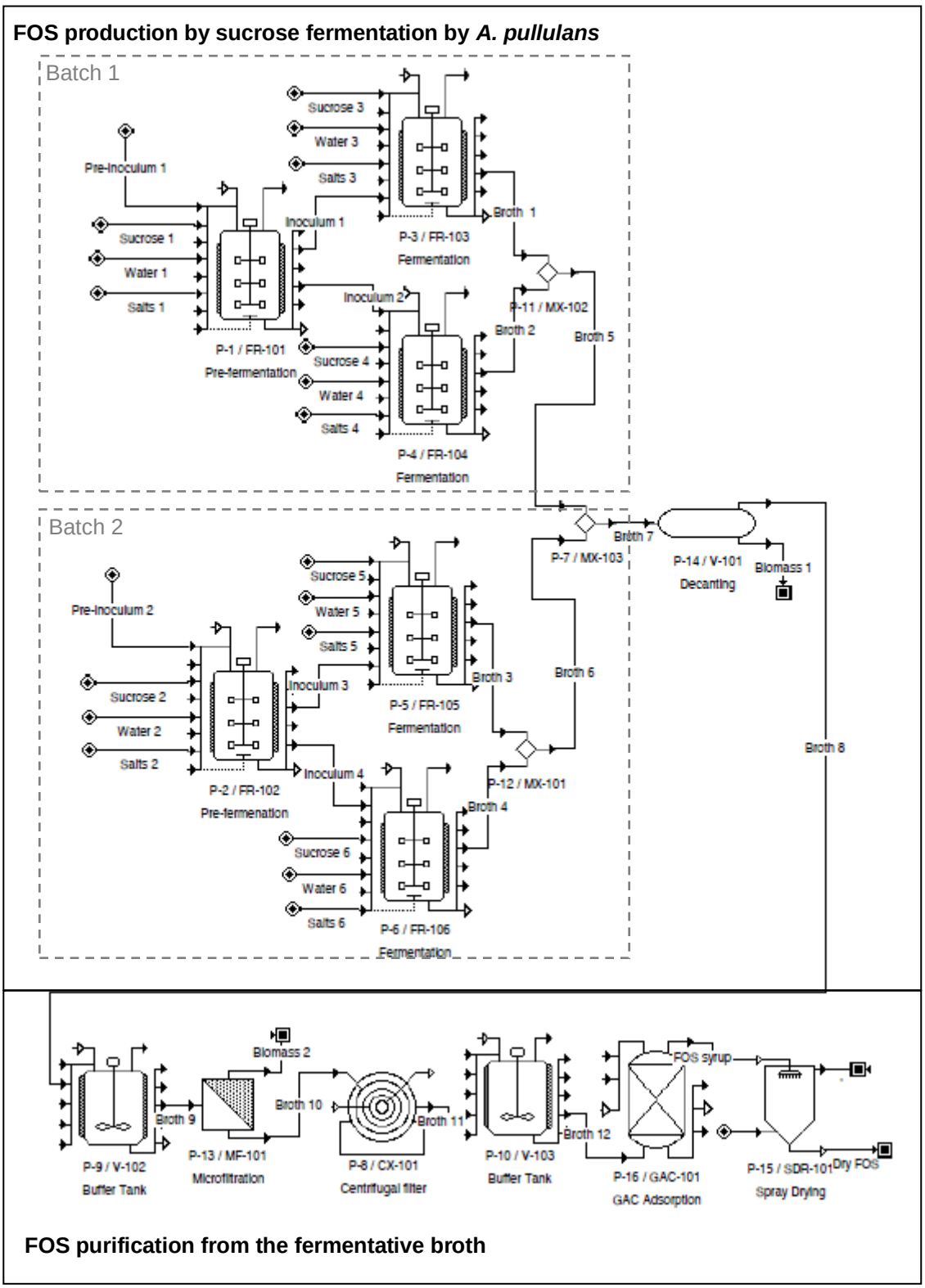


Figure 6.4. Process flowsheet of FOS industrial production implemented in SuperPro-Designer software.

Process Economics

The analysis of the process economics was conducted taking into account the equipment investment costs for an industrial unit to produce 10.000 ton/year of FOS. To determine the project cash-flow, the investment, operation and financing costs were considered, as well as the revenue obtained by FOS sales. The total investment estimated was 20 M€, which considers equipment, pumps, valves, measurement equipment and facilities costs. The operation costs accounted for the raw material cost for pre-fermentation and fermentation processes (sucrose, water and salts), purification step (activated charcoal and ethanol), energy consumption (electricity and heat), labor (5 persons), maintenance, insurance, laboratory and quality control. The project financing was studied considering the use of borrowed capital, with an interest rate of 8 %, being 70 % of the investment paid in 6 years with 2 years grace period, and the remaining 30 % paid in 5 years with a grace period of 1 year. The amortization costs were also considered for the lifetime of the project (17 years: 2 years for building the industrial unit and 15 operating years – average lifetime of the equipment). For the calculation of the project liquid cash-flow, a CIT (Corporate Income Tax) plus "DERRAMA" (Municipal tax) taxes of 26,5 % were considered. For the determination of the project NPV (Net Present Value) and IRR (Internal Rate of Return), a discount rate of 10 % was applied. The ROI (Return on Investment) was also calculated. The revenue was estimated with two net price values of FOS, 3,0 €/kg and 3,5 €/kg. The price assumption was set based on UBIC Consulting reports in The World Prebiotic Ingredient Market of 2008 . An inflation rate of 3 % per year was used. The evaluation of the process economics is presented in Table 6.1.

Table 6.1. Process economics (investment, operational cost, FOS net price, NPV, IRR and ROI) for a FOS production industry of 10.000 ton/year, based on a one-stage FOS production process.

Project investment	20.000.000,00 €	20.000.000,00 €
Operational cost	1,98 €/kg_{FOS}	1,98 €/kg_{FOS}
FOS net price	3,5 €/kg	3,0 €/kg
NPV	71.491.389,88 €	41.564.720,03 €
IRR	45,18%	32,80%
ROI	5 years	6 years

6.4. DISCUSSION

FOS has emerged as one of the most important candidates in the functional food market. (Sangeetha *et al.* 2005). In view of the great demand for FOS as food ingredients, scope exists for screening and identification of newer strains capable of producing FTase and/or FFase, as well as for improving and optimize FOS production processes.

Therefore, the current study focused on the scale-up of the optimized one-stage FOS production process from sucrose fermentation by *A. pullulans*. The process developed and optimized on Chapter 3 was scaled 25 times, presenting similar fermentation kinetics to the ones obtained in the lab scale experiments. A similar FOS production yield and process productivity of 66,5 % ($g_{FOS}/g_{sucrose}$) and 3,10 ± 0,00 g_{FOS}/L.h, respectively) were also obtained suggesting that the process developed has potential to be used at an industrial level. Furthermore, FOS composition in the maximum production time point in the semi-pilot fermentation was the same as the one obtained in the lab scale experiments (68 % 1-kestose and 32 % nystose).

In the present study, a process design and evaluation of the process economics for a FOS industry with a production capacity of 10.000 ton/year, based on the one-stage FOS production process developed in this thesis (Chapter 3 and present Chapter) and the FOS purification process by activated charcoal as described by Nobre *et al.* (2012), was developed. A total investment of 20 M€ was considered and a production cost of 1,98 €/kgFOS was determined. The raw materials cost for sucrose fermentation by *A. pullulans* represents 72 % of the total operational cost, within which, the cost of sucrose, with a share of 63 %, was by far the largest one. FOS purification step cost, namely activated charcoal and ethanol, corresponds to almost 14 % of the total operational cost. Vaňková *et al.* (2008) reported a total investment cost for a FOS production unit, based on the two-stage process, to be 5,031 M€ with an operational cost of 2,26 €/kg_{FOS} in powder. Although the reported investment cost is 25 % of the total investment cost considered in this study, the production cost is 12 % higher. A FOS industrial unit is a very attractive business, considering that it presents a NPV of 71,5 M€ with a high IRR of 45,18 %, with a FOS net price of 3,5 €/kgFOS. The ROI of the investment is expected to be 5 years, 2 of which are the unit construction and assembly. Decreasing 0,5 €/kgFOS in the FOS net value, a decrease of 30 M€ in NPV and 13 % in the IRR are expected but the business still presents very profitable numbers. The threat of this business is the incorporation of this product in the global market. In 1995, the global market for FOS from sucrose was estimated to be 20.000 tons per year (Menrad 2003). UBIC Consulting reports in The World Prebiotic Ingredient Market of 2008 estimated a FOS global market of 24.100 ton, having FOS from sugar market an estimated volume of 9.640 ton, so a 10.000 tons/year production industry represents the entire global market of FOS produced via sucrose. Nevertheless, in China, Baolingbao Biology have a FOS production plant with an installed production capacity of 40.000 ton/year, which may be indicative of a potential increasing trend in the FOS global market in the years to come.

6.5. CONCLUSION

In summary, increasing 25 times the scale of the process, similar FOS production yield, productivity, composition and kinetics were obtained, thus suggesting that the one-stage FOS production process by sucrose fermentation by *A. pullulans* has potential to be used at an industrial level, being competitive with the current usual industrial two-stage FOS production, by reducing operational costs.

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CHAPTER 7:
GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK



"Live as if you were to die tomorrow. Learn as if you were to live forever."

Mahatma Gandhi.

The major conclusions drawn from the thesis are addressed in this chapter. Furthermore, suggestions and recommendations for further research in this field are discussed.

7.1. CONCLUSIONS

7.2. RECOMMENDATIONS FOR FUTURE WORK

7.1. CONCLUSIONS

The aim of the this thesis was to optimize the fermentation conditions for the production of prebiotics, namely FOS, in order to develop a new one-stage production process that would be more competitive, technically and economically, than the current two-stage industrial production process. To achieve this global aim, several studies were conducted and some methodologies were successfully developed and implemented. The main drawn from the work developed are the following:

- Based on a presumptive and indirect colorimetric plate assay herein developed for the evaluation of transfructosylation activity in fungi, several fungal strains from the *Aureobasidium*, *Aspergillus* and *Penicillium* species from Micoteca da Universidade do Minho (MUM) collection were found to be FOS-producing microorganisms. *P. aurantiogriseum*, *P. chrysogenum*, *P. commune*, *P. corylophilum*, *P. crustosum*, *P. expansum* and *P. roqueforti*, not previously reported as FOS-producing microorganisms, were found to have high transfructosylation activity for FOS production, thus have a great potential for FOS production. Additionally, a new *Aspergilus* strain, the *A. ibericus*, was found to be a FOS-producing microorganism.

- Experimental factorial design and response surface methodology allowed the determination of optimal fermentation conditions for maximizing FOS production yield by *Aureobasidium pullulans*, that were found to be T = 32 ºC, agitation speed = 385 rpm. The validity of the model was proven by fitting the values of the variables in the model equation and carrying out the fermentation at those values of the variables. It was achieved a 64,1 ±0,0 % ($g_{FOS}/g_{sucrose}$) total FOS production yield and a process productivity of 2,67 ± 0,05 g_{FOS}/L.h, within 48 h of fermentation. When compared to the conventional two-stage process, an increase in the production yield was achieved, making this study an important contribution in the development of more efficient, less expensive processes for the industrial production of FOS.

- The location of the enzymes with transfructosylation activity in the extracellular, periplasmic and intracellular spaces of *A. pullulans* was done. The higher transfructosylation activity (U_t = 22,71 U.mL^{-1}) was found for the enzyme(s) present in the extracellular space, thus supporting the idea of developing a one-stage FOS production process, whereas the higher U_t/U_h ratio (1,18) was obtained for the enzyme(s) present in the intracellular space.

- Fed-batch fermentation of sucrose, supplied by a linear feeding profile, by *A. pullulans*, allowed a slight increase in FOS production yield, when compared to the batch production process, of 1,3 % ($g_{FOS}/g_{sucrose}$). Nevertheless, the productivity in fed-batch operation mode was found to be three times lower than in batch mode. Moreover, with the fed-batch operation mode, fructofuranosyl nystose (GF4) was produced, which can add commercial interest to the FOS mixture obtained.

- The 25 times volume scale-up of the one-stage FOS production process confirmed the potential use, at an industrial level, of the sucrose fermentation process developed and optimized in this thesis. Moreover, the operational cost of an industrial unit producing 10.000 ton/year of FOS, based on the one-stage process, was found to be lower than the current two-stage industrial process.

- An estimated investment of 20 M€ in a FOS production industrial plant, based on the one-stage production process, can achieve a NPV of 71,5 M€, with a IRR of 45,18 % and ROI of 5 years, representing a very attractive business opportunity.

7.2. RECOMMENDATIONS FOR FUTURE WORK

The results achieved in this work raised interesting perspectives on the development of a new one-stage FOS production process that can be an alternative to the conventional two-stage industrial process. The issues resulting from the developed work suggest future directions in FOS production research, as follows:

- Explore the fungi identified as having high transfructosylation activity in order to ascertain their potencial to produce FOS, by cultivating these microorganisms in sucrose liquid medium.

- Implement strategies to increase FOS production yield and productivity in one stage-process by glucose removal from the fermentation medium with glucose oxidase;

- Thoroughly study the FOS-producing enzymes from *A. pullulans* CCY 27-1-94 in order to optimize the reaction conditions and favour the transfructosylation activity over the hydrolyzing activity;

- Extend the fed-batch strategy for FOS production, with a view to obtain a FOS product with higher composition of fructofuranosyl nystose (GF4), which can have a higher value in the market, by optimization of the linear feeding strategie in order to increase the process productivity.

- Considering that the FOS production process herein developed and optimized was only validated using a scale increase of 25 times, and since the the volumes to be used at industrial level are up to 250 m^3, further studies are needed to confirm the industrial applicability of the process, such as, for example, mass transfreance studies, use of alternative feedstocks (byproducts of other industries) and optimization of the downstream process.

Overall, innovative and valuable results were obtained in FOS production and relevant issues were raised for the continued research in this topic.